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The Vital Story of
MACARONI* ENRICHMENT

by Science Writer

This is the fourth article in a series devoted to the story of cereal enrichment

Dramatic results have been recorded about the value of enrichment in improving health. From the United States, the Bataan peninsula in the Philippines, Newfoundland and many other parts of the world comes word of the great benefits which result from enrichment.

For years, some forward-looking manufacturers of macaroni and noodle products have used enrichment to make their good foods better. They know that enrichment restores important vitamin and mineral values which are unavoidably lost in milling, and they recognize their responsibility to provide the greatest health-building benefits for the public.

Enrichment is really a simple process. It adds the following essential elements to the food during manufacture.

Thiamine—also called vitamin B_1. This vitamin helps to build physical and mental health. It is essential for normal appetite, intestinal activity and sound nerves.

Riboflavin—also called vitamin B_2. This vitamin helps to keep body tissues healthy and to maintain proper function of the eyes. It is essential for growth.

Niacin—another "B" vitamin, is needed for healthy body tissues. Its use in the American diet has done much to make a serious disease called pellagra disappear.

Iron—is a mineral used in all enrichment. It is essential for making good, red blood and preventing nutritional anemia.

Products made from semolina may be enriched by two methods. One uses small square wafers which contain all the vitamins and iron necessary to enrich 100 lbs. The wafers break up in a small amount of water which is then added to the paste. For manufacturers who use the continuous press method, a powdered concentrate of the vitamins and iron, called a premix, is available. This is added by a mechanical feeding device.

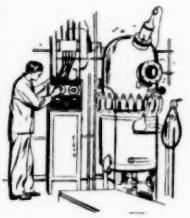
**Macaroni is used here in the generic sense. It includes all alimentary pastes: macaroni, spaghetti, pastina, noodles.*

These are the minimum and maximum levels, in milligrams per pound, required by the Federal Definitions and Standards of Identity for enriched alimentary pastes.

	Min.	*Max.*
Thiamine (vitamin B_1)	**4.0**	**5.0**
Riboflavin (vitamin B_2)	**1.7**	**2.2**
Niacin	**27.0**	**34.0**
Iron	**13.0**	**16.5**

NOTE: These levels allow for 30% to 50% losses in kitchen procedures.

Nowadays scientists are able to "build" duplicates of many of Nature's essential complexes in the laboratory. This has happened with many vitamins. First the chemical composition is learned and the pure substance is isolated. Then a "duplicate" is made which is identical chemically and biologically with Nature's product. A vitamin is a vitamin regardless of its source just as salt is salt whether it comes from a mine or is evaporated from the sea. So efficient is large scale manufacturing that vitamins are sold at a lower cost than if they were extracted from natural sources.



The Hoffmann-La Roche people, who produce a good percent of the vitamins used in enrichment, use amazingly complex processes with scientific production controls. This requires modern, special equipment filling whole buildings, each one a city block square and many stories high.

The combination of scientific research, thorough know-how and mammoth manufacturing processes—plus the far-sightedness of leaders in the macaroni industry—is helping vitally to make good macaroni products better.

This article, reprints of which are available without charge, is published as a service to the macaroni industry by the Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 286 St. Paul Street, West, Montreal, Quebec.

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B&L Bausch & Lomb **REFRACTOMETERS**

FACTORS AFFECTING RETENTION OF B VITAMINS IN CORN BREAD MADE WITH ENRICHED MEAL[a]

I. THE RELATION OF pH TO THE RETENTION OF THIAMINE, RIBOFLAVIN, AND NIACIN IN CORN BREAD

JUNE K. PACE AND JESSIE WHITACRE
Department of Rural Home Research, Agricultural Experiment Station, College Station, Texas

(Manuscript received October 3, 1952)

It has been shown in a previous study *(11)* that retention of B vitamins in corn bread made with low and moderate proportions of leavening (pH of batter 5.4 to 6.2) ranged from 93 to 102% for riboflavin, from 95 to 98% for niacin, and from 84 to 89% for thiamine. But with high proportion of leavening (pH of batter 6.6 and 6.7) riboflavin fell somewhat (to 89%), niacin increased slightly (to 108%), and thiamine retention decreased to only 7%. It therefore appears that at some pH between 6.2 and 6.6 for corn bread batter, there is a critical point, previously unknown, at which thiamine is rapidly destroyed during baking. A laboratory study of the relation of pH to thiamine retention seemed desirable from both theoretical and practical considerations.

PROCEDURE

The non-enriched degermed meal was obtained from a milling company in Texas, and either Clemson or Merck[b] premix was used to enrich part of the meal in the laboratory. Other ingredients for the corn breads were bought at local groceries except one of the baking powders which the manufacturer supplied. The manner of enriching and the standardized recipes used are described in the report of the previous study on enrichment of corn meal and grits *(11)*.

Two sets of experiments have been carried out. In one set, the variable was the pH of the batter in a series of corn breads made by the standardized sour milk recipe. The variable in the other set of experiments was the type of baking powder as the only leavening.

In the breads in which pH was the variable, thiamine was the only vitamin determined. Beginning with the proportion of leavening in the standard recipe (1.9 g. soda to 375 g. milk along with 4.5 g. baking powder to 250 g. meal) the amount of soda was increased by different amounts until a point of complete destruction of thiamine was reached. The standard proportion of leavening *(11)* was considered the logical lowest level for this series because breads made with low leavening (1 g. soda and 1.5 g. baking powder, pH 5.4 and 5.5) showed approximately the same average retention (89.1%) as the standard sour milk bread (89.3%).

The part of baking powder in producing change in pH value was investigated. The baking powder was omitted in one of a pair of some of the corn bread loaves made with the high amounts of soda. Also 2 sour milk breads were made with the standard amount of soda (1.9 g.) as the only leavening; and 2 sweet milk loaves with excessive baking powder (18 g.) and no soda.

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[a] Published with the approval of the Director of the Texas Agricultural Experiment Station as technical article 1623.

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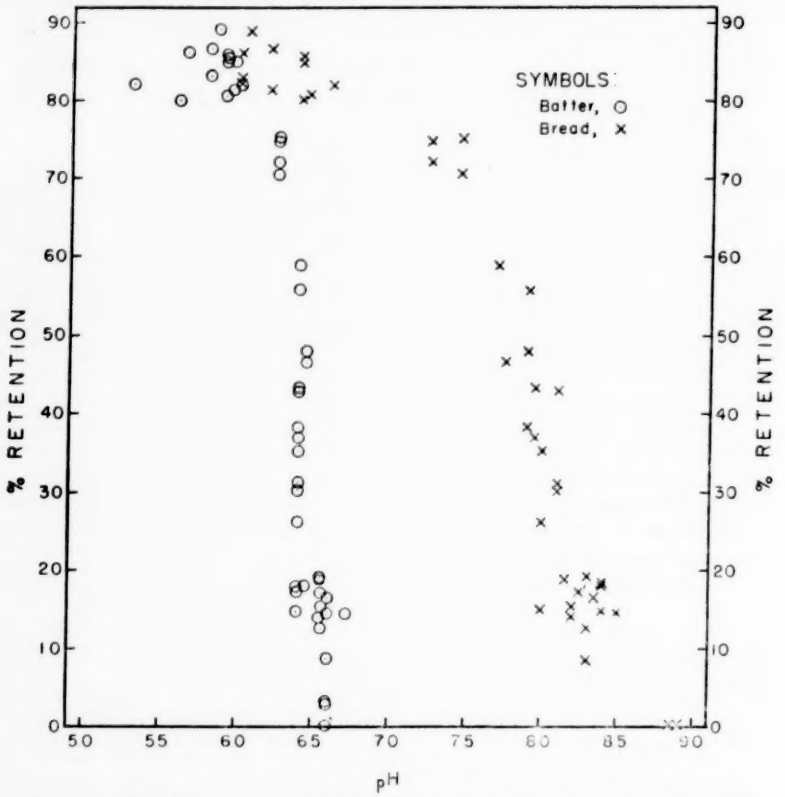

Figure 1. Thiamine retention in relation to pH of batter and of bread. Each retention is represented by two symbols, one for batter, the other for bread. The two corresponding symbols are on the same horizontal line. The pH was not taken for 6 breads, one each from batter with pH of 5.8 and of 5.95, 4 from batters with pH of 6.6.

In the breads baked with different types of baking powder, thiamine, riboflavin, and niacin were determined. These breads were made by the standard sweet milk recipe. Royal, Calumet, and Rumford baking powders[b] represented, respectively, the tartrate, the S.A.S. phosphate, and phosphate types. On finding that each type, regardless of proportion used, gave a characteristic pH in the batter, it was decided to use the level called for in the standard recipe, 4.5 g. to 250 g. of meal. One loaf of corn bread with each type of baking powder was baked on each of 5 days.

An adaptation of Hennessy's thiochrome method was used for thiamine *(6)*, of the microbiological method of Snell and Strong for riboflavin, and of Snell and Wright for niacin, as detailed in *Methods of Vitamin Assay (10)*. A standardized routine was followed in determination of pH values by means of a Beckman pH meter. The sample of batter was removed just before placing batter in the baking utensil. The pH was taken within a few minutes time. Bread was prepared for pH determination by adding 25 ml. of water to 5 g. of finely crumbled bread.

Duplicate determinations were made on each sample of batter and of bread. The duplicates of each pair were averaged. With few exceptions the meal used was analyzed each day.

RESULTS AND DISCUSSION

The consistent results obtained on the meal served as a check on analytical procedure. Other checks were determination of a known amount of a vitamin added to a meal sample, and the recovery of the pure vitamin used as a sample. Values found for supplemented samples were: for thiamine average 93%, range 89 to 99%; riboflavin average 99%, range 95 to 103%; niacin average 104%, range 99 to 108%. Values found for pure vitamins were: for thiamine average 98%, range 92 to 101%; riboflavin average 104%, range 96 to 112%; niacin average 101%, range 93 to 112%.

TABLE 1

Thiamine content of corn bread batters of different pH values

pH of batter	Corn meal					
	Batch a		Batch b		Batch c	
	Number of determinations	Range of thiamine content	Number of determinations	Range of thiamine content	Number of determinations	Range of thiamine content
		µg. per g.		*µg. per g.*		*µg. per g.*
6.4 and 6.45	4	1.23 to 1.36	8	1.05 to 1.18	4	1.24 to 1.31
Critical zone						
5.3 to 6.0	None		4	1.09 to 1.12	4	1.20 to 1.30
6.55	4	1.29 to 1.32	None		None	
6.6	2	1.26 to 1.29	6	1.09 to 1.18	None	

pH as the variable. The scatter diagram (Figure 1) shows the percentage retention in relation to pH of batter and of bread. Here it is apparent that the highest thiamine retentions (80 to 89%) were obtained when the pH of the batter did not exceed 6.0 and the pH of the resultant bread was not over 6.6. With each successive increase in pH of batter the rise in pH during baking became greater, and the retention less. However, with pH of batter at 6.4 and 6.45 there was a markedly greater variation in retention than at any other point. Coefficients of variability were 26.6 and 59.8%, or 8 times and 18 times the variability at pH of 5.3 to 5.95. Among the undetermined factors which caused the widely different retentions at pH 6.4 and 6.45 may have been a variable rate in the chemical reaction of the leavening during baking. The thiamine content of the batters with pH of 6.4 and 6.45 closely resembled the thiamine content of batters at other pH values both lower and higher, when the corn meal used was from the same enriched batch. Table 1 shows that for each of 3 batches of meal, thiamine content of batters at pH 6.4 and 6.45 was essentially the same as at pH values below 6.0, and at 6.55 and 6.6. The wide differences in retention in the critical pH zone (6.4 and 6.45) must be attributed to changes which occurred during baking. Negligible retention or complete destruction of thiamine accompanied the baking of batters with pH of 6.6.

Figure 1 further makes clear that with pH of batters 6.0 and below, the pH of breads remained on the acid side. But with pH of batters 6.25 and above, pH values of breads were on the alkaline side.

The summary of thiamine retentions in relation to the different amounts of soda and the associated pH values of batter and of bread is given in

Table 2. Statistical analysis by group comparison *(9)* for the breads made with 1.9 and 2.5 g. of soda showed that difference in retention (average 85 and 83%, respectively) was not significant. Difference between the group of breads with 2.5 g. of soda (retention 83%) and those with 3.5 g. (retention 73%) was highly significant. The coefficient of variability for the retention of thiamine in corn breads made with 1.9 g. soda was 3.3%; with 2.5 g. soda, 3.6%; and with 3.5 g. soda, 2.9%.

TABLE 2

Retention of thiamine in corn bread in relation to pH of batter and of bread

Amount of soda	Number of replications	pH of batter		pH of bread		Retention	
		Range	Mean	Range	Mean	Range	Mean
grams						%	%
1.9	7	5.3-5.95	5.75	6.0-6.2	6.08[c]	82-89	85
2.5	4	5.6-5.9	5.82	6.4-6.45	6.41	80-86	83
3.5	4	6.25 only	6.25	7.25-7.45	7.35	71-75	73
4.5	10	6.4 only	6.4	7.7-8.1	7.97	26-59	40
5.0	6	6.4-6.45	6.42	7.75-8.4	8.18	13-48	27
5.5	2	6.55 only	6.55	8.15-8.3	8.22	19 only	19
5.7	8	6.55-6.6	6.57	8.0-8.5	8.26	9-17	14
6.0	6	6.6 only	6.6	8.85-8.9[d]	8.88	0-3	0.5

[c] For 6 breads; pH not taken for 1 bread.
[d] For 2 breads; pH not taken for others.

The findings are of practical importance in connection with an analysis of the proportion of ingredients used in homemade corn bread. Out of 74 recipes from Texas homes, 29 of them called for more soda than in the standard recipe which, as has been pointed out, gave the highest thiamine retention. The soda in 11 of these recipes was comparable with those amounts which gave the lower retentions, ranging downward from 73 to 40%. With excess of soda the benefit of thiamine addition to corn meal may be largely lost.

Table 3 shows that the pH of the batter was the same whether or not baking powder was used. The pH of the bread without baking powder was consistently slightly lower than of the mate loaf baked with baking powder on the same day. However, all loaves had an alkaline pH, and thiamine retentions in each pair of loaves were the same or showed negligible differences. The buttermilk and eggs could have had little influence on change of pH of batter because pH of egg ranged from 7.4 to 7.7, mode 7.5, and of milk from 4.3 to 4.5, mode 4.4. The results of using each chemical leavening agent alone give further evidence that the diminishing retentions should be attributed to increasingly excessive amounts of soda.

The retentions with each leavening agent used alone (Table 3) are similar to those in breads containing the standard proportion of both, 1.9 g. soda and 4.5 g. baking powder, and pH ranging from 5.3 to 5.95 (Table 2). Soda alone was used in the standard proportion (1.9 g. to 375 g. buttermilk, pH of batter 5.8). The baking powder when used alone was an excessive proportion (18 g. to 250 g. meal, 4 times the standard, and pH 5.95 and 6.0). Apparently the baking powder was so well balanced

as to prevent the pH rising to the neutral point and thus the excessive baking powder did not increase the amount of thiamine destroyed during baking. Lowering of quality was noted in the corn bread with excessive baking powder—intensified yellow color, coarse grain, and harsh texture.

Three types of baking powder. The data for the corn breads made with the 3 types of baking powder are summarized in Table 4. The pH values of batters were remarkably consistent and also characteristic within each type of baking powder. The modal pH was 6.0 for Royal, 6.2 for Rumford, and 6.35 for Calumet. The pH values of the breads were also strikingly similar within baking powder type.

TABLE 3

Thiamine retention in corn bread as affected by different amounts of soda with and without a constant amount of baking powder and by each leavening used alone

Soda	pH of batter		pH of bread		Retention	
	With baking powder*	Without baking powder	With baking powder	Without baking powder	With baking powder	Without baking powder
grams					%	%
4.5	6.4	6.4	7.9	7.7	56	59
5.0	6.45	6.45	7.9	7.75	48	47
5.5	6.55	6.55	8.3	8.15	19	19
5.7	6.55	6.55	8.3	8.2	13	14
5.7	6.6	6.55	8.35	8.25	16	17
6.0	6.6	6.6	No data	No data	3	3
6.0	6.6	6.6	No data	No data	0	0
6.0	6.6	6.6	8.9	8.85	0	0
1.9		5.8		No data		87
1.9		5.8		6.0		83
None	5.95†		No data		83	
None	6.0†		6.6		82	

*Standard amount (4.5 g./250 g. of meal) when used with soda.
†Excessive amount of baking powder (18 g.) the only leavening.

Retentions of 95 and 96% for niacin and of 102% for riboflavin indicate that the baking process had little or no effect on these vitamins. The mean retentions of thiamine by breads leavened with the 3 baking powders fell within a narrow range. The small variation in thiamine retention is shown by the standard error of each mean (Table 4). Yet within this narrow range it is to be noted that the group with lowest pH of batter (6.0) had the highest retention, 85%. Variance analysis showed a significant difference between the three baking powders. However, from a practical dietary viewpoint, with retentions at the high levels found, choice among these brands of baking powders may be based on other considerations than the thiamine retentions associated with each baking powder.

The consistent and characteristic results with corn bread leavened with each type of baking powder are in line with Farrer's findings *(5)* for destruction of thiamine when boiled in a buffer solution. For any given buffer there was a linear relationship between rate of thiamine destruction and the pH of the boiling solution. The relationship was different for each buffer and the slope of the curve changed as the ionic constitution of the solution was altered.

TABLE 4

Retention of thiamine, riboflavin, and niacin in corn bread [a] as affected by kind of baking powder

Kind of baking powder	Range of pH		Thiamine			Riboflavin			Niacin		
	Batter	Bread	Number of bakings	Retention		Number of bakings	Retention		Number of bakings	Retention	
				Range	Mean ± SE		Range	Mean		Range	Mean
				%	%		%	%		%	%
Calumet (S. A. S. phosphate)	6.35-6.40	6.6 only	5	80-85	83.0±1.0		No data	No data	2	93-100	96.3
Royal (tartrate)	6.00-6.05	6.4-6.5	5	82-89	85.1±1.2	2	98-107	102.4	2	93-98	95.1
Rumford (phosphate)	6.20-6.30	6.5-6.6	5	81-82	81.7±0.2		No data	No data	2	93-99	95.8

[a] Standard sweet milk recipe, 4.5 g. baking powder to 250 g. meal.

The only publication found on retention of B vitamins in corn bread is that of Hoffmann-La Roche Laboratories *(7)*. Their corn bread retained from 89 to 92% of the thiamine whether made with enriched bolted meal or unenriched whole corn meal. A moderate amount of leavening was used.

The findings in the Texas study with corn bread are concordant with results reported for biscuits, cake, and muffins. McKim and Moss *(8)* found a rise in pH during the baking of biscuit dough and cake batter, and they noted that the differences between raw and baked products varied with the type of leavening used. Barackman *(2)*, Williams and Fieger *(12)*, and Briant and Hutchins *(3)* found decrease in thiamine retention in biscuits with increase in pH, as did Briant and Klosterman *(4)* for muffins; and Arny and Hanning *(1)* for both biscuits and muffins. Of these studies with other products, the one most nearly resembling the Texas study is that of Williams and Fieger. They used the same proportion of different types of leavening, found little difference in the thiamine content of enriched biscuit, but a definitely descending order in which tartrate baking powder stood ahead of phosphate and S.A.S.-phosphate.

SUMMARY

Increasing the amount of soda, from 1.9 to 6.0 g. in the standardized sour milk recipe for corn bread produced successively larger values in pH of batter and of bread, but successively smaller thiamine retentions. With pH of batters 6.0 and below, the pH of corresponding breads did not exceed 6.6 and retentions were good (80 to 89%). With pH of batter 6.25 to 6.6, the pH of the resultant breads ranged from 7.2 to 8.9 and retentions fell precipitately. Negligible retention or complete destruction of thiamine followed the baking of batters with pH of 6.6. A moderate amount of soda (1.9 g. per 375 g. of buttermilk) used alone or combined with the standard amount of baking powder (4.5 g. to 250 g. meal) gave good retention. Similar good retentions were obtained with excessive amount (18 g.) of baking powder used alone. Evidently, a proper balance of the baking powder precluded destruction of the thiamine in the batter while it was baking.

With each of three types of baking powder to make corn bread, there was little if any destruction of riboflavin and niacin. Thiamine retentions were good—with tartrate baking powder 85%, S.A.S-phosphate 83%, and phosphate 82%. Highest retention was associated with lowest pH of batter (6.0). Differences in these thiamine retentions are too small to be taken into account when choosing among the three brands of baking powder.

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LITERATURE CITED

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FACTORS AFFECTING RETENTION OF B VITAMINS IN CORN BREAD MADE WITH ENRICHED MEAL.[a]

II. RELATION OF CRUST AND CRUMB AND OF BAKING UTENSIL TO RETENTION OF THIAMINE IN CORN BREAD

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(Manuscript received Oct. 3, 1952)

There is a great diversity in type of pans used for baking corn bread batter in Texas homes. That fact and consideration of the important place of corn bread in the diet of Southern people prompted this study to determine the thiamine retention when loaves were baked in different typical pans and when the batch of batter was divided to make muffins and corn sticks.

PROCEDURE

The corn meal was obtained as a degermed non-enriched product. The meal was enriched in the laboratory with Clemson premix.

Corn breads baked in different pans were made according to the standardized sour milk recipe which is described in another report *(4)*. Pans for loaves had similar area of bottom and were from 1⅛ to 1⅞ inches deep. There were two round layer cake pans, one tin, one aluminum, a rectangular pyrex baking dish, and a No. 6 iron skillet. Some batters were divided and baked as muffins in tin or aluminum pans in sets of six, and in iron corn stick pans. Bottom diameter of muffin tins was 2¼ inches, top 2½ inches, depth 1⅝ inches. The corn stick pan was divided into 22 sections each 3¼ inches long × ⅞ inch wide at the top.

The same oven was used for all bakings, one utensil at a time, placed always in the same central position. Baking time was 30 minutes at 215°C.(419°F.) for all products. Baking periods of 25 minutes were tried for muffins and corn sticks, but laboratory workers decided that the 30-minute baking gave products more nearly comparable with loaves in appearance and eating quality. Four bakings were made in each of the seven types of utensils.

The loaves in the skillet were baked in connection with the study of the relation of pH of corn bread batter to thiamine retention of bread *(3)*. Otherwise, on each day, batters were baked in three types of utensils. The order of using tin, aluminum, and pyrex for loaves, and of tin, aluminum, and iron for divided batters was rotated.

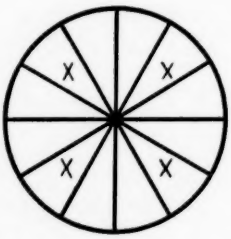
The relation of crust and crumb to thiamine retention was studied in two ways, by analysis separately of crust and crumb of corn bread loaves and by comparison of products which differed in proportion of crust and crumb. For study of the separated crust and crumb, the material for determination of thiamine was taken from some of the loaves which were sampled and analyzed in the usual way. A sharp knife was used to cut the entire layer of crust off of the crumb, the depth of crust being judged by the laboratory worker. Each portion—crust and crumb—was weighed separately, grated, then put through a 16-mesh wire strainer by pressure with a spoon, and lastly stirred with a few strokes of the spoon.

Samples for batter were removed before pouring batter into baking pan and were weighed within a few minutes. The plan of sampling baked products which was always

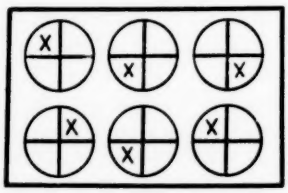
[a] Published with the approval of the Director of Texas Agricultural Experiment Station as technical article 1622.

followed is shown by diagram in Figure 1. For rectangular loaves and muffins, the representative portions together approximated ¼ of the baked product, for round loaves ⅛, and for corn sticks ½ of each baking. These portions from each baked product were finely divided and well mixed in the manner stated for crust and crumb. This procedure for one product at a time was carried out as quickly as possible and subsamples for analysis were weighed immediately thereafter.

Thiamine was determined by an adaptation of Hennessy's method *(3)*. Duplicate determinations were made on each sample of batter and of bread, and each pair of duplicates averaged. Retentions were calculated on the total batch moist basis.

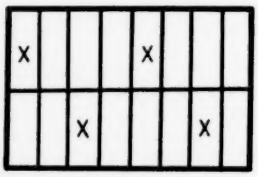


Represents 3 utensils for corn bread loaf

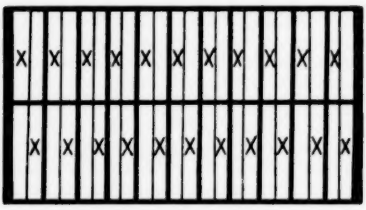


Tin and aluminum pans for corn meal muffins

	Bottom diam.	Ht.
No. 6 iron skillet	7 3/4"	1 7/8"
Aluminum layer cake pan	8"	1 1/4"
Tin layer cake pan	8 1/4"	1 1/8"



Pyrex pan for corn bread loaf



Iron pan for corn sticks

Figure 1. Diagram of plan for sampling corn bread loaves corn muffins, and corn sticks. The portions marked with an x were taken to prepare for subsamples.

RESULTS AND DISCUSSION

Crust and crumb of loaves. The data for comparison of crust and crumb of corn bread loaves are shown in Table 1. That satisfactory results were obtained with separate analysis of crust and crumb is indicated by the close agreement (last column of Table 1) between the sum of these two parts and the thiamine content of the same bread intact. The slightly greater total thiamine in the bread intact in 3 of 4 replications, is probably due mainly to the additional loss of water while preparing the crust and crumb sample. The values for crust as a percentage of loaf weight and of

TABLE 1

Content of thiamine in crust and crumb in corn bread loaves

Determined value				Crust as portion of		Sum: crust and crumb, thiamine per batch	Bread intact, determined, thiamine per batch	Difference in total thiamine: bread intact minus sum of crust and crumb[c]
Crust		Crumb						
Weight	Thiamine	Weight	Thiamine	Grams per batch[b]	Thiamine per batch			
grams	*μg./g.*	*grams*	*μg./g.*	%	%	*μg.*	*μg.*	%
159.6	1.08	355.4	0.87	31	36	481	496	3.1
149.8	1.14	363.2	0.93	29	34	509	520	2.2
152.0	1.00	372.0	0.75	29	35	432	445	3.0
160.8	1.02	351.2	0.76	31	38	430	426	−0.9
				Av. 30	Av. 36			

[b] A batch is the entire amount of batter and bread in one baking. Each horizontal line of data represents one baking.

[c] Total micrograms in sum of crust and crumb = 100%.

thiamine content fall within a narrow range—weight from 29 to 31 and thiamine from 34 to 38. No attempt was made to calculate retention for crust and crumb separately as there is no way of knowing what portion of the batter went into the crust and what remained in the crumb. The percentage of thiamine in the crust was calculated thus:

$$\frac{\text{total thiamine in crust}}{\text{total thiamine in crust + crumb}} \times 100.$$

The proportions of weight and thiamine content represented by the crust as reported here are applicable only to corn bread loaves made by the standardized recipe and baked in the same sized utensil as used in this study. Excessive soda (4.5 and 5.0 g. to 250 g. meal), which is reported elsewhere to have reduced thiamine in the whole loaf *(3)* was found to increase the proportion of thiamine in the crust to 48% compared to 36% when the standard amount of soda was used. The association of lower total retention with a greater proportion of crust in the baked product is shown in the results of dividing a batch of batter for baking.

TABLE 2

Thiamine retention in corn bread baked in different utensils

Product	Number of bakings	Kind of pan	Surface area per batch[d]	Crust as a part of loaf weight	Retention mean ± SE
			sq. in.	%	%
Loaves	4	Tin	126	No data	83±1
	4	Aluminum	120	No data	84±2
	4	Pyrex	125	No data	87±2
	4	Iron	112	30	86±1
Muffins	4	Tin	141	37	81±2
	4	Aluminum	141	42	76±2
Sticks	4	Iron	269	68	66±1

[d] The amount of product from the full recipe.

Different types of utensils for baking corn bread batter. The results with the corn bread baked in each type of pan were very consistent. Coefficients of variability for the retentions on different days were: for loaves baked in tin 3.5%, in aluminum 4.5%, in pyrex 3.9%, and in iron 3.4%; for muffins in tin 4.5%, in aluminum 5.6%; and for sticks in an iron pan 2.5%. The pH values in all 28 bakings were favorable to retentions; pH of batters ranged from 5.8 to 5.9, and of baked products from 5.9 to 6.2.

The data are summarized in Table 2. Surface areas of loaves were similar, since bottom areas of baking utensils were similar. A batch of muffins with the same amount of batter as for one corn bread loaf had a 17% greater surface area than the average surface of loaves. This was true whether the muffins were baked in aluminum or tin pans. A batch of batter baked as sticks had a surface area more than twice as great as the loaves. Retentions consistently were greater as surface area and proportion of crust were less (Table 2).

The results of analysis of variance for the thiamine retention in all of the baked products follow.

Item	Source of variance	df	Sum of squares	Mean square	F	
					Error term: Item 5	Error term: Item 6
1	Total	27	1,493			
2	Groups of products*	2	1,188	594.00	54.696†	48.689†
3	Pan material for loaves	3	37	12.33	1.135 N.S.	
4	Pan material for muffins	1	40	40.00	3.683 N.S.	
5	Error	21	228	10.86		
6	Combined items 3, 4 and 5 for error term	25	305	12.20		

*Loaves, muffins, and sticks.
†Significant at 1% level; N.S., not significant.

Here it is shown that the retentions of thiamine by breads baked in pans of different materials did not differ significantly. Neither was there a statistically significant difference in retention in muffins baked in aluminum and tin. In contrast, for the groups of products distinguished by size and shape, loaves, muffins, sticks, differences in thiamine retention were highly significant. This is true whether the single or the combined error term is used to test for significance.

The absolute differences between groups of baked products in percentage retention—loaves vs. muffins 6, loaves vs. sticks 19, muffins vs. sticks 13—are large enough to be of practical importance in diet planning and evaluation. The smaller the portions into which a batch of batter was divided for baking, the greater the surface area, and the greater the proportion of weight in the crust, but the less of thiamine retention.

That the smaller the portion of raw mixture used for baking of a quick bread, is associated with less retention of thiamine, has been reported also by Barackman *(1)*. Biscuits retained 88% of the thiamine when the dough was rolled to ⅜-inch thickness, but only 79% when rolled ¼-inch thick "to obtain a thin crusty biscuit." This author found no difference in the

thiamine content of biscuits due to varying the baking time from 9 to 17 minutes. It appears unlikely, therefore, that the trial corn muffins baked for 25 minutes contained more thiamine than those baked for 30 minutes to provide data.

Both similarity and difference are found between results in baking corn bread batter and yeast leavened bread dough. A single reference—the study of Zaehringer and Personius *(5)*—is cited since their findings concerning relation of crust to thiamine retention confirm those of five earlier studies on yeast bread. The results with corn bread are concordant with those on yeast bread in that thiamine retention is less as proportion of crust is greater. The corn meal and yeast products differ in that corn muffins and sticks retained less thiamine than did the corn bread loaves (really layers), whereas yeast rolls retained more thiamine than the yeast bread loaves. The same baking time was required to obtain characteristic acceptable corn products. Sticks were crisp and crusty; muffins and loaves were of approximately the same height and with crust comparable in color and thickness. Thus proportion of crust and crumb was the only variable affecting retention in the corn meal products. But to obtain acceptable products from yeast dough required that the rolls be baked only half as long as the loaves. Thus there were 2 variables affecting retention in the yeast leavened products. Zaehringer and Personius conclude that shorter exposure to heat compensated in the rolls for their greater surface, compared with a loaf of bread.

SUMMARY AND CONCLUSIONS

Corn bread loaves made by the standardized sour milk recipe had an average of 30% of their weight and 36% of their thiamine content in the crust. When batters were divided for baking, the total surface area of the batch and the proportion of crust increased, but the percentage retention of thiamine decreased. The mean retention in loaves, muffins, and sticks was respectively 85%, 79%, and 66%, with corresponding proportions of crust 30%, 40%, and 68%.

Variance analysis showed that there were no statistically significant differences in thiamine retention among the loaves baked in tin, aluminum, pyrex, and iron utensils. Retentions by muffins baked in tin and aluminum pans did not differ significantly. But for the three groups of baked products, the differences in mean thiamine retention were highly significant.

The differences in thiamine content of loaves, muffins, and corn sticks is large enough to be of practical importance. Where the dietary supply of thiamine is a problem, corn bread loaves are preferable to muffins, and muffins preferable to corn sticks.

Acknowledgments

The authors sincerely appreciate the cooperation of the Quaker Oats Co. in making available degermed non-enriched meal, and of the South Carolina Experiment Station in sending small quantities of Premix when requested. They are grateful to the Subsistence Department of the Texas A & M College for permitting use of an institutional size mixer to enrich the meal. They express gratitude to Miss Helen M. Forsthoff for assistance in the laboratory and for painstaking care in preparing the manuscript.

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FACTORS AFFECTING RETENTION OF B VITAMINS IN CORN BREAD MADE WITH ENRICHED MEAL.[a]

III. RETENTION OF THIAMINE IN CORN MUFFINS MADE WITH COMMERCIAL MIXES AND IN CORN BREAD MADE WITH SELF-RISING MEALS

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Prepared mixes for baked products have met with ready acceptance as time and labor savers in home cooking. The growing number of mixes now includes some in which corn meal is the main ingredient. Self-rising enriched corn meal also has recently appeared on the market. Corn meal is an important item in the nation's food supply, especially in the South. For these reasons it seemed desirable to use corn muffin mixes and self-rising meals in a study of the retention of thiamine in corn meal when cooked for human consumption.

PROCEDURE

Five brands of corn muffin mix and one brand each of whole and bolted self-rising meal were included in the study. Two packages of each brand of mix were used, each to make one batch of 6 muffins.

The muffins were made according to the directions on the package. The only instructions on the bags of self-rising meals were: "Use your favorite recipe. Omit salt, soda, baking powder." Because corn bread of equally good quality and similar thiamine retention had resulted from use of the standardized recipes *(4)*, one calling for sweet milk and baking powder, the other for sour milk, soda, and baking powder, each standardized recipe was used with omission of salt, soda, and baking powder, in corn bread loaves made with the self-rising meals. Two different brands of mixes were used on a given day. Routinely, a sweet milk and a sour milk self-rising corn bread loaf were made on the same day. Each batch of batter, whether for a loaf or muffins, was baked one at a time, in the same oven at 215°C.(419°F.) for 30 minutes, except that for 2 brands of mixes the time was reduced to 25 minutes to avoid over browning. For comparison with products from the commercial preparations, muffins and corn bread were made with bolted meal which had been enriched in the laboratory.

Sampling was done as previously described *(3)*, a portion from each quarter of a loaf and ¼ of each muffin were taken as the material for subsamples. Thiamine was determined by an adaptation of Hennessy's thiochrome method *(1)* in each of the mixes, meals, batters, and baked products. Duplicate determinations were run on each sample and each pair of duplicates was averaged. Retentions were calculated on the moist basis for each total batch of batter and the resultant baked product.

RESULTS AND DISCUSSION

The content of thiamine ($\mu g/g.$) in each mix and meal is shown in Table 1. Only mix 1 contained enriched meal; mixes 2 and 3 employed enriched flour along with non-enriched meal. The content of thiamine of

[a] Published with the approval of the Director of the Texas Agricultural Experiment Station as Technical Article 1621.

the mixes and meals varied markedly, the highest value being about ten times the lowest. For the most part enrichment made the difference. The thiamine content of one package of mix 2 slightly exceeded the minimum federal standard. All others were below federal standard, although the second package of mix 2 and the bolted self-rising meal were only slightly below.

Average weight of muffins from mixes 1, 2, and 3 ranged from 48.6 to 49.4 g., while those from mixes 4 and 5 weighed approximately ¾ as much. Quality of baked products from the bolted self-rising meal and from mixes 2, 3, and 4 were rated good. Muffins from mix 1 and 5 were too crumbly; those from mix 1 were compact, and had dry surface; those from mix 5 were bitter. Corn bread from the whole self-rising meal was judged of low quality due chiefly to off flavor.

Data on thiamine content and retention in muffins are also given in Table 1. As with the raw mixes, the mix batters showed pronounced differences in total thiamine content. Thiamine in muffins differed even more than in the batters, due to the variation in destruction of thiamine during baking. The mix muffins show 3 levels of retention—2 brands of 73

TABLE 1

Thiamine content and retention in corn meal muffins made with commercial mixes and with meal enriched in laboratory [c]

Number	Thiamine in mixes and meals	Thiamine per batch [d] moist basis		Retention		pH	
		Batter	Muffins	Per batch	Average	Batter	Muffins
			Mixes				
	μg/g.	μg	μg	%	%		
1	3.43	586	437	75	73	6.3	....
	3.14	525	374	71		6.25	6.8
2	4.03	888	695	78	74	6.5	6.6
	5.27	949	655	69		6.4	6.6
3	.....	528	264	50	53	6.5	7.0
	2.46	523	285	55		6.5	6.9
4	0.32	112	66	59	55	6.6	6.8
	0.41	105	52	50		6.6	6.8
5	0.80	98	30	31	31	6.55	7.5
	0.82	103	31	30		6.55	7.5
		Meal, degermed, enriched in laboratory [e]					
	μg/g.	μg	μg	%	%		
1	4.01	273	227	83	84	5.8	6.1
	3.86	267	225	84		5.9	6.0
	4.37						
2	3.52	438	334	76	79	5.8	6.0
	3.51	376	300	81		5.8	6.0
	3.70						

Federal Standards: minimum, 4.41 μg./g.; maximum, 6.62 μg/g.

[c] Each horizontal line of data represents a separate baking.

[d] The entire amount of batter and muffins from one package of mix.

[e] The two meals were different batches and enriched with different enriching mixtures. The standard sour milk corn bread recipe was used for muffins made with each meal.

and 74%, 2 of 53 and 55%, and one brand of 31%. These levels were associated with differences in pH values of batters and of muffins. The highest retention accompanied the lowest pH of batters together with pH of muffins below the neutral point. For mix 3 (retention 53%) the pH of the batter was higher than for mix 1 and similar to mix 2; but the pH of the muffins from mix 3 rose to a higher value than those from mixes 1 and 2. In the batter of mix 4 (retention 55%) pH was the highest of all, but rise during baking left muffins still slightly on the acid side. With mix 5, pH of batter was only slightly less than of mix 4, but the change while baking elevated the pH of mix 5 to an alkaline value, resulting in lowest retention of any mix.

Other than for mix 3, the leavening agent is not stated except that it is a baking powder. However, comparison with retention and pH of batters and muffins made of meal enriched in the laboratory shows that the baking powders in the mixes were less favorable to thiamine retention than were the leavening ingredients in the standard recipe—tartrate baking powder, soda, and sour milk. Also three of the mixes had a much greater change in pH during baking than did the standard recipe muffins. Apparently the acid and alkaline ingredients of the baking powder in mixes 1, 3, and 5 were not balanced to give the best effect during baking.

Table 2 gives the data which permit comparison of the self-rising corn meal loaves with those of meals enriched in the laboratory, also of sweet milk and sour milk loaves.

TABLE 2

Thiamine content and retention in corn bread made with self-rising enriched meal and meal enriched in laboratory [1]

Kind of meal	Thiamine per batch, moist basis				Retention		pH			
	Batter		Bread							
	Sweet milk	Sour milk	Sweet milk	Sour milk	Sweet milk	Sour milk	Batter with sweet milk	Bread with sweet milk	Batter with sour milk	Bread with sour milk
					Meal, self-rising, enriched					
	μg	μg	μg	μg	%	%				
Bolted,	1,027	997	491	816	48	82	6.45	7.35	5.95	6.1
de-	1,034	993	465	815	45	82	6.5	7.4	5.9	6.1
germed	1,071	1,019	493	874	46	86	6.5	7.4	6.0	6.05
					Av. 46.3	Av. 83.3				
	884	901	601	705	68	78	6.4	7.1	5.8	5.9
Whole	899	863	643	711	72	82	6.3	7.0	5.8	6.0
	932	904	628	720	67	80	6.35	6.95	5.9	6.0
					Av. 69.0	Av. 80.0				
					Meal enriched in laboratory					
	μg	μg	μg	μg	%	%				
	1,216	1,162	1,019	1,012	84	87	6.05	6.4	5.9	6.2
	1,140	1,200	1,016	1,030	89	86	6.0	6.5	5.9	6.2
De-	1,240	1,167	1,021	971	82	83	6.0	6.4	5.9	6.2
germed	1,127	1,076	949	914	84	85	6.0	6.5	5.8	6.1
	1,181	.....	1,016	.....	86	....	6.0	6.5	....	....
					Av. 85.0	Av. 85.1				

[1] Each horizontal line of data represents a separate baking.

The content of thiamine, total micrograms per batch, was about the same in the sweet milk and the sour milk batters with the 3 kinds of meal. But thiamine contents of the sweet milk and sour milk breads were similar only for meal enriched in the laboratory. With self-rising whole meals, the sour milk breads averaged 14% higher in thiamine than the sweet milk, while with bolted self-rising meals the sour milk breads were nearly twice as rich in thiamine as were those made with sweet milk.

There are clear cut similarities and differences in thiamine retention (Table 2). Self-rising breads made with sour milk and the laboratory enriched breads with either sweet or sour milk had good retentions which range from 78 to 89%, and average 84%. Variance analysis of these four groups of bread showed that the differences in retention were not statistically significant. From the practical viewpoint, difference between 80 and 85% retention is of little dietary importance. In contrast, the sweet milk breads with whole meal (average retention 69%) and the sweet milk breads with bolted meal (average retention 46%) differed significantly from each other, and both groups had significantly less thiamine than the 4 groups with good retention.

In the 15 breads with good retention, the pH of the batters ranged from 5.8 to 6.05. The pH values of the resultant breads varied from 5.9 to 6.5. The lower retentions in breads made with sweet milk and either bolted or whole self-rising meal were associated with the higher pH values, which range from 6.3 to 6.5 for the batters and from 6.95 to 7.4 for the breads. The low retention breads had a considerably greater change in pH value during baking than did the breads with high retention. Apparently in the sour milk breads, the lactic acid of the sour milk and phosphate together neutralized the soda during the baking process. In the sweet milk breads, however, there was an excess of soda, which reacted with water and yielded sodium hydroxide. The sodium hydroxide produced a rise in pH and increased the destruction of thiamine during baking. With these particular self-rising meals, the direction should be to use sour milk.

The findings in a previous study *(2)* indicated that the pH of corn bread batter should not exceed 6.0, since with such pH values the higher pH reached during baking is still on the acid side and thiamine retention uniformly good. The leavening ingredients of self-rising meals and commercial mixes could be made to conform to those limits of pH values. Then such preparations would be not only time savers in food preparation, but in addition an important means of improving the human diet where corn meal is an important foodstuff.

SUMMARY AND CONCLUSIONS

Enriched cereal in some, but not others, of 5 brands of corn muffin mix accounted for most of the differences in thiamine content of the batters made with them; but difference among the resultant muffins was due more to differences in loss of thiamine while baking. Three levels of retention were found—73 and 74% for 2 brands, 53 and 55% for 2 others, and 31% for one brand. Muffins of meal enriched in the laboratory retained 79 and 84% of the thiamine.

Self-rising corn bread made with sour milk showed good retention for both whole meal (80%) and bolted meal (83%) values comparable with the 85% retention by corn bread from meal enriched in the laboratory. When made with sweet milk, self-rising whole meal breads retained only 69% of the thiamine, and bolted meal breads only 46%.

All good retentions were associated with acid pH values in both batter and bread; poor retentions with higher pH of batters and pH of bread at or above the neutral point.

With enrichment of cereal and with leavening such as to favor best retention of thiamine, corn muffin mixes and self-rising meals would be welcome time savers in food preparation, and a means of improving human diets.

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NUTRITIVE REQUIREMENTS OF ACETOBACTER. I. VITAMIN REQUIREMENTS OF *ACETOBACTER XYLINUM*[a]

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In a consideration of factors which influence the efficiency of the conversion of ethyl alcohol to acetic acid in the production of vinegar, understanding the specific nutrient requirements of the various species of Acetobacter concerned is important. Although considerable data have been reported for *Acetobacter suboxydans*, little is known regarding the specific nutrient requirements of *Acetobacter xylinum*. In the present discussion, the preparation of a basal medium for determining the nutritive requirements of *A. xylinum* and observations on the vitamin requirements of this organism are presented.

In an investigation of the growth requirements of *A. suboxydans*, Underkofler, Bantz, and Peterson *(3)* found that in addition to suitable carbon source, organic nitrogen, and mineral salts, the medium must also contain pantothenic acid, p-aminobenzoic acid, and nicotinic acid. The amino acids required by *A. suboxydans* for growth were determined by Stokes and Larsen *(2)*. In a review of the vitamin and growth factors required by bacteria, Peterson and Peterson *(1)* indicated that biotin and riboflavin were synthesized by this organism.

EXPERIMENTAL

Organism and inoculum. The culture of *A. xylinum*,[b] was carried on slants of yeast extract agar (0.5% Difco yeast extract, 0.3% KH_2PO_4, 2% glucose and 1.5% agar, pH 6.2).

Inocula were obtained from 24-hour slants which had been transferred daily for at least 3 days. The cells were removed from the agar slant with 10 ml. of sterile physiological saline (0.85%) and were washed 3 times. The washed cells were finally suspended in 10 ml. of saline. One-tenth ml. of this suspension was used to inoculate 10 ml. of the test medium in a 125-ml. Erlenmeyer flask. The inoculated flasks were incubated at 30°C. for 48 hours. They were shaken continuously in a shaking machine, during incubation, to provide aeration.

Determination of growth. Because of the slimy film growth of the organism the measurement of growth on a basis of turbidity was impossible. Growth was measured by acid production after 48 hours of incubation. The original 10 ml. of medium were titrated to pH 6.0 with 0.1 N NaOH. Because of the nature of the growth no attempt was made to correlate the amount of acid produced with actual cell count.

Media. The basal medium employed was essentially a modification of that described by Underkofler, Bantz, and Peterson (3). It contained per 100 ml.: Difco vitamin free casamino acids, 0.5; dextrose, 3.0; adenine sulfate, 0.002; guanine hydrochloride, 0.002; uracil, 0.002; K_2HPO_4, 0.05; KH_2PO_4, 0.05; $MgSO_4 \cdot 7H_2O$, 0.02; NaCl, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; and $MnSO_4 \cdot 3H_2O$, 0.001; grams, respectively. The pH was adjusted to 6.0. The growth factor employed was made up in aqueous solution, with the exception of

[a]Contribution No. 882 from Massachusetts Agricultural Experiment Station, Department of Bacteriology and Public Health and Department of Food Technology, Amherst.

[b]Obtained from the National Institute of Health.

adenine sulfate, guanine hydrochloride, and uracil, from crystalline compounds and were stored at 4.0°C. The latter 3 substances were acidified with HCl in order to effect solution. All media were sterilized in steam for 15 minutes at 121°C. before use. In preliminary experiments 3 sources of vitamin-free hydrolyzed casein (Difco vitamin-free casamino acids, G.B.I. vitamin-free casein hydrolysate, and a vitamin-free casein hydrolysate prepared in the laboratory) showed no difference in growth stimulation. Difco vitamin-free casamino acid was used as the source of amino acid in most of the experiments. The growth factors or vitamins studied were added to the basal medium in concentrations of micrograms per 100 ml. as follows: Calcium pantothenate, 20; folic acid, 20; l-inositol, 20; niacin amide, 20; p-aminobenzoic acid (PABA), 20; pyridoxine hydrochloride, 40; riboflavin, 40; thiamin hydrochloride, 20; and biotin, 0.5.

Transfers in test medium. Because of the nature of the growth, it was difficult to wash the cells of the inoculum sufficiently to remove all traces of growth factors carried over from the yeast extract agar. To reduce the effect of these carry over substances to a minimum the organism was passed through several series of test media until little or no growth could be detected in the basal medium. In carrying out this procedure, each succeeding flask of medium was inoculated with a standard loop of clear liquid from a 24-hour culture in the preceding flask. It was found that from 3 to 5 such transfers were necessary to eliminate the stimulatory effect of carry-over growth factors.

Glassware. All glassware (pipettes, flasks, beakers, volumetric glassware, etc.) were washed with a detergent and then acid-cleaned with chromic acid cleaning solution. They were rinsed 6 times in single distilled water and then rinsed twice in double distilled water.

Purine and pyrimidine bases. To determine the necessity of adenine, guanine, and uracil for growth, *A. xylinum* was inoculated into the basal medium plus vitamins with and without the addition of the above compounds. Throughout 4 transfers in the experimental media it was evident that the addition of adenine sulfate, guanine hydrochloride, and uracil caused a marked stimulation of acid production; henceforth, the basal medium contains these compounds.

Vitamin requirements. The various vitamins under consideration were added to the basal medium singly and in combination. The pertinent results, summarized in Table 1, indicate the essentiality of PABA for growth and acid production by *A. xylinum*. Varying concentrations of PABA were added to the basal medium to determine the quantitative requirements of *A. xylinum* for this nutrient (see Table 2).

DISCUSSION

As already indicated, the presence of adenine, guanine, and uracil appeared to be necessary for optimum acid production for *A. xylinum* under the conditions employed. Then these compounds were considered as essential components of the basal medium employed in determining the vitamin requirements of this organism.

TABLE 1

Effect of vitamins on acid production by A. xylinum

Medium	Vitamins added							
Basal medium	x	x	x	x	x	x	x	x
p-Amino benzoic acid		x	x	x	x	x	x	
Calcium pantothenate			x	x	x	x	x	x
Riboflavin				x	x	x	x	x
Niacin amide					x	x	x	x
Thiamine hydrochloride					x	x	x	x
Biotin						x	x	x
Pyridoxine hydrochloride						x	x	x
Folic acid							x	x
l-Inositol							x	x
Acid production[a]	0.5	10.1	10.9	9.8	10.0	10.0	8.3	1.4

[a] Number of ml. of 0.1 N NaOH to titrate 10 ml. of test media. Results reported were obtained after 48 hours of incubation in the third set of test media.

TABLE 2

Effect of concentration of PABA on acid production by A. xylinum

Milli gammas PABA/10 ml. medium	Ml. 0.1 NaOH required for titration after 48 hours
0.0	0.3
0.5	0.3
5.0	0.4
10.0	4.3
50.0	6.3
100.0	6.8
1,000.0	8.0

Experiments set up to determine the vitamins required for growth and acid production were carried out using calcium pantothenate, folic acid, l-inositol, niacin amide, PABA, pyridoxine hydrochloride, riboflavin, thiamin hydrochloride, and broth alone and in combination. As indicated in Table 1, *A. xylinum*, only in the presence of PABA, was able to produce marked amounts of acid, with or without the other vitamins. Little or no stimulation of acid production was imparted by the other vitamins. On a quantitative basis (see Table 2) at least 10 milli gammas per 10 ml. of medium of PABA were required for a significant degree of acid production. Upwards of 1,000 milli gammas per 10 ml. were required for maximum acid production.

CONCLUSIONS

Of calcium pantothenate, folic acid, l-inositol, niacin amide, PABA, pyridoxine hydrochloride, riboflavin, thiamin hydrochloride, and biotin, only PABA was found to be essential for growth and acid production by *A. xylinum*. Little or no stimulatory effect was observed in the other vitamins.

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DETECTION OF HORSEMEAT IN MEAT PRODUCTS BY A MODIFIED PASCHKE METHOD[a]

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In 1938, Paschke *(23)* introduced a method for detection of horsemeat in admixture with beef, mutton, or pork, based on the observation that horse fat is richer in linolenic acid than are the depot fats of other large domestic animals. According to this procedure, the hexabromide number of the mixed fatty acids is used as a measure of the percentage of linolenic acid in the fat extracted from the meat sample under investigation. Variations of the Paschke procedure have been used by Fisher *(14)*, Crowell *(9)*, Dalley *(10)*, and by Hynds *(19, 20)*. These workers report hexabromide values ranging from 4.1 to 21.5 for horsefat, and from 0.1 to 1.0 for beef, mutton, and pork fats. The chief difficulty mentioned by these authors is the poor reproducibility of the hexabromide method. Hynds *(20)* found that hexabromide values from 1.4 to 4.0 were obtained when identical mixtures of horsemeat and beef were analyzed by several independent workers, using the same procedure.

This paper describes a modified Paschke procedure, whereby ultraviolet spectrophotometry is used to determine the percentage of linolenic acid in the fat extracted from meats and sausages. In recent years, this method has generally supplanted other procedures for analysis of fats for polyunsaturated acids, particularly in instances where a high degree of accuracy is required.

EXPERIMENTAL METHODS

The experimental work forming the basis of this paper included a study of extraction procedures in order to arrive at a standard analytical procedure which gives precise results for the percentage of linolenic acid in the fat obtained from undried meat, and the application of this procedure to meats and sausages.

Preliminary studies. Moderately lean chuck meat from U. S. inspected beef (sample 4), and lean horse flank meat (sample 10) were used to determine the effects of cooking and of extraction conditions on the percentage of linolenic acid found in the extracted fat. About 500 g. of each material was passed 3 times through a hand-operated food chopper, which is equipped with a cutter blade, and which has an extrusion plate with ⅛-inch perforations. The meat was quartered and kneaded after each passage through the chopper.

Approximately 25 g. of the chopped beef or horsemeat was used for each extraction study. Single-thickness thimbles were used for the Soxhlet extractions; the samples to be cooked were weighed in the thimbles, and the filled thimbles were placed for one hour in an air oven maintained at 90°C. Thus, the percentages of extracted fat given in Table 1 are based on the weights of the original uncooked and undried samples. The extractions were carried out in all-glass equipment, using diethyl ether or petroleum ether (b. 30-60°C.) for either 3 or 5 hours. The heating rate was adjusted so that siphoning occurred 12 to 15 times per hour.

Extractions were also carried out by maceration of the chopped meat samples with ice and solvent in a Waring blendor. The blendor jar was charged with about 50 ml.

[a]Sponsored in part by the Cudahy Packing Company, Omaha, Nebraska.

of chloroform, carbon tetrachloride, or petroleum ether (b. 30-60°C.), an equal volume of cracked ice, and the weighed chopped meat sample. Agitation was carried out until the solvent began to boil. After the mixture had cooled, the solvent layer was removed, using a separatory funnel, and the aqueous layer was returned to the blendor jar. The process was repeated twice. Emulsions were always formed in this method of extraction; those obtained when carbon tetrachloride was used as solvent separated readily, but the character of these mixtures made it difficult to decide when complete separation had occurred. Attempts to use diethyl ether as solvent resulted in stable emulsions, as was usually the case when petroleum ether was tried.

TABLE 1

Effects of extraction conditions on the percentage of linolenic acid found in the fat from cooked or uncooked beef or horsemeat

Sample	Method of extraction	Extracted fat	
		Found	Linolenic acid
		%	%
Beef (sample 4)			
Uncooked	Soxhlet, petroleum ether, 3 hours	9.08	0.51
	Soxhlet, petroleum ether, 5 hours	10.02	0.60
	Soxhlet, petroleum ether, 5 hours	10.03	0.58
	Soxhlet, diethyl ether, 5 hours	7.68	0.39
	Waring blendor, chloroform	4.60	0.41
	Waring blendor, carbon tetrachloride	9.61	0.53
Cooked[b]	Soxhlet, petroleum ether, 3 hours	8.20	0.53
	Soxhlet, petroleum ether, 5 hours	10.02	0.56
	Soxhlet, petroleum ether, 5 hours	10.17	0.55
	Waring blendor, petroleum ether	7.75	0.48
	Waring blendor, chloroform	8.85	0.42
	Waring blendor, carbon tetrachloride	10.48	0.56
	Waring blendor, carbon tetrachloride	10.50	0.53
Horsemeat (sample 10)			
Uncooked	Soxhlet, petroleum ether, 3 hours	10.20	9.55
	Soxhlet, petroleum ether, 5 hours	11.35	9.57
	Soxhlet, petroleum ether, 5 hours	4.94	6.50
	Waring blendor, chloroform	8.65	7.94
	Waring blendor, carbon tetrachloride	10.56	9.75
Cooked[b]	Soxhlet, petroleum ether, 3 hours	10.78	9.67
	Soxhlet, diethyl ether, 5 hours	11.41	9.58
	Waring blendor, carbon tetrachloride	11.20	9.89

[b] Weighed samples of ground meat were cooked in thimbles or beakers for one hour at 90°C. before extraction.

The extracts obtained by either method of extraction were dried overnight, using anhydrous sodium sulfate, then filtered into a weighed 250-ml. flask. All apparatus was rinsed with fresh dry solvent, and the washings were combined with the corresponding filtrates. After the bulk of the solvent had been distilled over a water bath, residual solvent was removed by a stream of dry nitrogen while the flask was warmed gently by an infra-red lamp. About two hours was required for complete removal of solvent in this manner; to insure this, however, the flasks were finally subjected to vacuum.

The percentages of linolenic acid in the fat extracts were determined by the ultraviolet spectrophotometric method of Brice, Swain, Schaeffer, and Ault *(3, 5)* using the revised constants reported by these authors at the 1948 Fall Meeting of the American Oil Chemists' Society *(28)*. Alkali-isomerization was accomplished by heating weighed samples of fat in 11% KOH-glycerol at 180°C. for 30 minutes; a blank was used for each set of 5 determinations. The solutions were diluted with absolute ethanol, and optical densities were measured with a Beckmann Model DU spectrophotometer. Meas-

urements were made at 262, 268, and 274 mμ and at 310, 316, and 322 mμ, in order to include corrections for background absorption and for any arachidonic acid which might be present. The dilutions were adjusted and the cell lengths were selected to give optical densities between 0.2 and 0.8 with slit widths between 0.5 and 0.8 mm. Conjugated dienoic and trienoic acids were also determined by the procedure of Brice and co-workers *(3, 4, 5)*, but no measurable amounts were found in any of the extracted fat samples.

Standard analytical procedure. Various meats and meat products were extracted, and the fat analyzed spectrophotometrically for linolenic acid. A procedure, found to give the most precise results in preliminary studies, was adopted as standard for these samples, and is summarized as follows:

With the exception of a sample of chili con carne, which was extracted directly, all materials were passed through the food chopper three times. About 25 g. of the uncooked, undried chopped sample was extracted at least 5 hours with petroleum ether (b. 30-60°C.); it was usually convenient to extract for 6 or 7 hours. It was advantageous to incorporate about 5 g. of anhydrous sodium sulfate before extraction in order to obtain clear, moisture-free extracts; when this was done, additional drying of the extract was usually unnecessary. Other details of the extraction and analytical procedures were the same as those used in the preliminary studies.

TABLE 2

Linolenic acid analyses of fat extracted from beef or horsemeat products

Sample no.	Identity	Extracted fat	
		Found	Linolenic acid
		%	%
1	Beef, U. S. inspected, chopped	8.4	0.47
2	Beef, U. S. inspected, round	.....	0.51
3	Beef tallow, packer's edible[c]	.....	0.48
4	Beef, U. S. inspected, chuck	10.0	0.59
5	Beef wieners, U. S. inspected	.....	0.74
6	Beef bologna, U. S. inspected	.....	0.74
7	Horse, fatty tissue	22.6	5.80
8	Horsemeat, round	.....	6.50
9	Horsemeat, very lean strip	2.4	9.05
10	Horsemeat, flank, lean	11.4	9.57
11	Horsemeat, lean	9.0	11.2
12	Horsemeat, very lean strip	6.3	14.7

[c] No extraction necessary.

This method was applied to 60 samples of meat or meat products. The samples listed in Table 2 were obtained from sources such that their identity cannot be questioned; in contrast, the materials listed in Table 3 were obtained from restaurants, markets, and similar outlets, and were submitted to the authors by regulatory agents as samples which were suspected of containing horsemeat. None of the samples examined was found to contain significant amounts of conjugated trienoic or tetraenoic acids.

RESULTS AND DISCUSSION

The primary purpose of the extraction studies made with known beef and horsemeat samples was to determine the conditions necessary for isolation of the representative sample of fat from a meat product. Judging from the results given in Table 1, this requirement can be met either by a 5-hour Soxhlet extraction, using low-boiling petroleum ether, or by the Waring blendor technique, using carbon tetrachloride as solvent. Although less time is required for each sample by the latter method, the operation requires continuous attention; several samples can be extracted, using a

battery of Soxhlet extractors, in less total elapsed time than by the blendor method. Hence, the 5-hour Soxhlet procedure, using petroleum ether (b. 30-60°C.) was adopted as standard for extraction of fat from various meat products.

For the beef sample studied, shorter extraction periods or use of diethyl ether as a solvent in the Soxhlet method resulted in inadequate fat removal, accompanied by low and erratic percentages of linolenic acid. This was also found when chloroform was used in the blendor method. The horsemeat extractions reached completion more rapidly than did the beef extractions. Pre-cooking the beef or horsemeat samples did not facilitate extraction, but the results obtained demonstrate that no significant change in the percentages of linolenic acid in beef or horsefat would occur under the heating conditions used for sausage manufacture.

TABLE 3

Linolenic acid analyses of fat extracted from meats or meat products suspected of containing horsemeat

Sample no.	Claimed to be:	Linolenic acid in extracted fat	
			%
13	Chopped beef		7.15**[d]
14-33	Chopped beef (20 samples)	Range	0.27 to 0.81
		Average	0.56
34-42	Beef trimmings (9 samples)	Range	0.35 to 0.63
		Average	0.50
43	Beef bologna		3.20**[d]
44	Beef bologna		2.76**[d]
45	Beef bologna		2.76**[d]
46	Beef bologna		1.91**[d]
47	Beef bologna		0.73
48	Beef bologna		0.71
49	Beef wieners		8.39**[d]
50	Beef wieners		4.41**[d]
51	Beef wieners		3.10**[d]
52	Beef wieners		1.34
53	Beef wieners		0.55
54	Beef salami		3.20**[d]
55	Beef salami		3.17**[d]
56	Mettwurst		0.58
57	Liver sausage		0.57
58	Summer sausage		0.83
59	Chili con carne		0.58
60	Corned beef hash		0.68

[d] ** Indicates it was concluded that these samples contained horsemeat.

The standard procedure of analysis for linolenic acid in the extracted fat was applied to 6 beef and to 6 horsemeat samples, with results as given in Table 2. Although there is considerable variation in the values obtained for each type of meat, the highest percentage of linolenic acid found in beef fat was 0.74 for sample 6, whereas the lowest value for horse fat was 5.80%, obtained with sample 7. This wide difference indicates not only that horsemeat and beef can be readily distinguished by this procedure, but that fairly low concentrations of horsemeat in beef can be detected.

The standard analytical procedure was applied to 48 samples of meats, sausages, and meat products. The percentages of linolenic acid found in the extracted fats are listed in Table 3. The results indicate clearly that samples 13, 43-46, 49-51, 54, and 55 (marked **) contained horsemeat. The salami samples, 54 and 55, are of particular interest. These were sections of sausage, submitted 4 months apart, and examined by 2 analysts, neither of whom was aware that the other sample existed. It was later learned that the 2 sections were parts of the same sausage and that independent evidence had established the fact that the sausage contained horsemeat.

Sample 52 was regarded as a borderline case; the authors did not believe that the 1.34% linolenic acid found in the extracted fat was sufficiently high to justify the conclusion that horsemeat was present, without careful consideration of the factors involved. In order to facilitate interpretation of this and similar results which might be obtained in the future, a thorough search of the literature was made to obtain information which could be used to estimate the allowances which should be made for the normal variation in the percentage of linolenic acid in beef fat, for the possibility that pork or mutton might be present in the sample under study, for errors in the spectrophotometric analysis, and for the possible fat contribution by cereals which are often used in sausage manufacture.

Table 4 shows published values for the percentages of linolenic acid in horse, ox, sheep, and pig depot fats. With one exception, these include the highest values reported for these animal fats. The abnormally high result of 11.4% linolenic acid found for "yellow" pork fat by Beadle, Wilder, and Kraybill *(2)* is not included. These workers, as well as Robinson and Coey *(26)* and Dugan, Petheram, and Kraybill *(12)*, remark that the occurrence of "yellow" pork fat is relatively rare, and that such carcasses are condemned at the slaughterhouse. This provides ample justification for concluding that pork of this type would not appear in an edible meat product, and therefore has no connection with the problem of horsemeat detection. In Table 4, the data reported by Paschke *(23)* and by Crowell *(9)* are included primarily because they were obtained in studies on the Paschke hexabromide method for detection of horsemeat.

The data given in Table 4 indicate that, although sheep fat is very low in linolenic acid, there is no significant difference in this respect between ox and pig depot fats. It is therefore concluded that the procedure outlined in this paper for the detection of horsemeat applied equally to beef, pork, or mutton products. The highest published value for the percentage of linolenic acid in beef, pork, or mutton fat is reported by Dugan, Maroney, and Petheram (11) for a sample of beef brisket fat; these workers obtained a result of 0.86% linolenic acid, using the spectrophotometric procedure of Mitchell, Kraybill, and Zscheile *(22)* as modified by Beadle and Kraybill *(1)*.

Using this value as a basis, the authors have adopted a figure of 1.10% linolenic acid as the maximum value which would be obtained by the standard procedure outlined in this paper, using an all-meat product composed of beef, pork, or mutton. The increase of 0.24 percentage units above the figure of 0.86 reported by Dugan *et al. (11)* is ample allowance for sys-

tematic errors and for differences in the procedures used for spectrophotometric analysis of the fat. Justification for this allowance is afforded by data given in the Report of the Spectroscopy Committee of the American Oil Chemists' Society *(24)* and by results presented by Brice, Swain, Herb, Nicholas, and Riemenschneider *(4)*. The former reference gives the results of a collaborative study, comparing, among others, the Brice and Swain procedure with the Beadle and Kraybill method; for samples of soybean oil containing about 8% linolenic acid, the total spread of all results by all methods was less than 15% of the average value. The deviations are presumably due to chance errors as well as to differences in the analytical procedures. Similar results were found by Brice *et al. (4)* in applying these and other procedures to samples of beef tallow and lard containing less tnan 1% linolenic acid.

TABLE 4

Published linolenic acid analyses of normal domestic animal fats

Animal	Location in animal body	Linolenic acid	Method	Reference
		%		
Horse	(a) Depot fat, U. S.	1.04	H	Crowell *(9)*
	(b) Depot fat	1.5	H	Paschke *(23)*
	(c) Body fat	2.0	H	Heiduschka & Steinrock *(16)*
	(d) Abdominal, Western range, U. S.	4.5	T	Schuette, Garvin, & Schwoegler *(27)*
	(e) Internal	17.1	S	Brooker & Shorland *(6)*
	(f) Mesenteric	16.0	S	Gupta & Hilditch *(15)*
	(g) Liver	25.0	S	Bruce & Shorland *(7)*
Ox	(h) Depot	None	F	Hilditch & Paul *(17)*
	(i) Depot	0.04	H	Crowell *(9)*
	(j) Depot	0.1	H	Paschke *(23)*
	(k) Ribs, (cooked)	0.42	S	Chang & Watts *(8)*
	(l) Rendered ruffle fat	0.45	S	Riemenschneider *et al. (25)*
	(m) Internal	0.46	S	Knight, Jordan, & Swern *(21)*
	(n) Beef tallow	0.56	SS	Brice *et al. (4)*
	(o) Brisket (17 animals, including cows, steers, yearlings)	0.86 (Highest) 0.60 av.	S	Dugan, Maroney, & Petheram *(11)*
Sheep	(p) Depot fat, Indian	None	S	Hilditch & Shrivastava *(18)*
	(q) Depot	0.1	H	Paschke *(23)*
	(r) Lamb chops (cooked)	0.68	S	Chang & Watts *(8)*
Pig	(s) Depot	0.10	H	Paschke *(23)*
	(t) Depot	0.14	H	Crowell *(9)*
	(u) Rendered lard	0.38	SS	Brice *et al. (4)*
	(v) Back fat of pigs fed exclusively on soybeans	0.5 av.	H	Ellis & Isbell *(13)*
	(w) Bacon (cooked)	0.67	S	Chang & Watts *(8)*
	(x) 9 normal pigs, abdominal	0.45 av.	S	Dugan, Petheram, & Kraybill *(12)*
	belly	0.72 av.		
	back	0.83 av.		

Legend for method:
F = Analysis based on fractional distillation of methyl esters of mixed fatty acids.
H = Analysis based on hexabromide value.
T = Analysis obtained by iodine number and thiocyanogen number.
S = Analysis by ultraviolet spectrophotometry.
SS = Highest result obtained by four ultraviolet spectrophotometric procedures, using the same fat sample.

For sausages and similar products, an allowance must also be made for the linolenic acid present in the oil contributed by cereal fillers, such as wheat, rice, oat, corn, or soybean flours. Of these, soybean flour would be most important. It is estimated, however, that the fat extracted from a beef, pork, or mutton sausage containing low-fat soy flour would contain no more than 1.50% linolenic acid. Higher values would occur only if horsemeat is also present. On this basis, the 1.34% linolenic acid found in the fat extracted from sample 52, Table 3, is not considered high enough to permit the conclusion that horsemeat was present.

SUMMARY

A modified Paschke method for detection of horsemeat in domestic meats, sausages, and other meat products is reported. The method depends on the observation that horse fat contains a higher percentage of linolenic acid than does the depot fat of normal oxen, sheep, and pigs. An extraction procedure is described which is reproducible, and the fats thus obtained yield precise results when analyzed spectrophotometrically for linolenic acid.

Specific criteria are given by which the percentage of linolenic acid found in the extracted fat can be interpreted in terms of the presence or absence of horsemeat. For all-meat samples, linolenic percentages higher than 1.10 are indicative of the presence of horsemeat. For sausages, this limit is extended to 1.50% linolenic acid. Ample allowance is made for factors which might cause abnormally high results to be obtained with samples free of horsemeat.

The method was applied to 6 samples of beef or beef tallow, to 6 samples of horsemeat or horse fat, and to 48 samples of chopped meat, sausages, and other products which were suspected of containing horsemeat.

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THE NUTRITIVE VALUE OF CENTRAL AMERICAN CORNS. I. NITROGEN, ETHER EXTRACT, CRUDE FIBER, AND MINERALS OF TWENTY-FOUR VARIETIES IN GUATEMALA[a]

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Corn (Zea mays) is the most important constituent of rural diets in Central America. In some indigenous families of the Guatemalan highlands it furnishes 80% of the calories and 70% of the protein *(14)*. Any change favorable or unfavorable in the nutritive value of local corn, and particularly in the quantity and quality of its protein will have far reaching effects in human nutrition in this area. Yet comparatively little is known about the range of variability in the content of essential nutrients that exists in the numerous varieties, races, and strains of corn consumed by the people.

Agricultural stations in Central America are at present attempting to develop higher yielding and more disease and drought resistant corns. Unfortunately there appears to be a tendency for such corns to have lower contents of important nutrients *(2)*. By including the most important of these nutrients in hybridization and selection efforts *(3, 4, 5, 11, 21, 23, 25)*, as well as by soil fertilization *(24)* and good soil management *(6, 27, 28)*, significant improvements in nutritive value can be obtained. When valuable genes associated with desirable nutritive qualities are lost through breeding for yield and resistance alone, as seems to have been done in the United States and elsewhere for many years, the task of improvement may be more difficult.

It was expected that the great diversity of corns cultivated in Central America must include some with more desirable nutritional characteristics than those in common use. If varieties of corn superior in nutritive value for each region could be found equal or superior in yield to those already in wide use, they could be increased and distributed as seed to effect an immediate improvement. Moreover, such corns could be further improved in nutritive value as well as yield through breeding *(8, 9)*. Such improvement could constitute an important contribution to the people directly dependent on a high corn diet.

[a]A cooperative study in which the corn samples and data relating to their origin were furnished by Dr. Irving E. Melhus, Director, Iowa State College Tropical Research Center, Antigua, Guatemala. The project was aided by a research grant from the Instituto de Fomento de la Producción (INFOP) Guatemala, Central America. Contribution I-22 from the Instituto de Nutrición de Centro América y Panamá.

This series of papers reports the biochemical studies carried out as part of such an improvement program. The first four describe the analysis of a group of representative corns selected to test the validity of this approach and to constitute plant material for a corn breeding program. The present paper is concerned primarily with the nitrogen content of these corns, although their moisture, crude fiber, ether extractable fraction, and minerals are also described. Subsequent papers concerning these corns will report their contents of (a) lysine and methionine; (b) tryptophane, niacin, thiamine, and riboflavin; (c) carotene. Studies of genetic and environmental influences on the nutritive value of specific corns are also in progress.

MATERIALS AND METHODS

The origin of each of the 24 varieties of corn studied is listed in Table 1. All were in common use in the region indicated. These corns were the most promising varieties found among over 400 grown in variety trials in 2 climatic zones, Antigua and Tiquisate. The varieties low in yield and quality of grain were eliminated in these trials.

The 2 Mexican corns were found growing in the vicinity of the villages named. The Venezuelan variety was developed at one of the Venezuelan Experiment Stations and distributed to corn growers. The variety TGY was a selection from a cross between two Cuban varieties. The variety 142–48 was a selection from the variety cross TGY and

TABLE 1

Some information concerning the twenty-four varieties of Latin American corns

Variety no.	Town	State or department	Altitude	Color	Group	Country	Year grown
			feet				
206-44⊗	Quezaltenango	Quezaltenango	7,500	W	Mountain	Guatemala	1949
159–44 # (2s)	Chichi.	El Quiché	6,800	Y	Mountain	Guatemala	1949
47A–46O.P.	Cobán	Alta Verapaz	4,200	W	Giganteum	Guatemala	1948
1470–45 #	San Sebastián	Huehuetenango	6,000	W	Giganteum	Guatemala	1948
25A–46⊗	Chichi.	El Quiché	5,200	Y	Giganteum	Guatemala	1948
92A–46 #⊗	Paraje Nuevo	Veracruz	.....	Y	Giganteum	Mexico	1948
192–44 #⊗	.....	.....	.....	Y	Giganteum	Guatemala	1948
15A–46O.P.	Villa Nueva	Guatemala	4,855	Y	Giganteum	Guatemala	1949
166–44O.P.	Los Pinos	Mexico	.....	W	Giganteum	Mexico	1949
31–44 #	Antigua	Sacatepéquez	5,000	Y	Giganteum	Guatemala	1949
20–47⊗	Mixco	Guatemala	5,000	Y	Giganteum	Guatemala	1949
1483–45⊗	Aguacatán	Huehuetenango	5,128	W	Giganteum	Guatemala	1949
21A–46B #	La Democracia	Escuintla	380	W	Coast	Guatemala	1947
10A–46O.P.	Chocolá	Suchitepéquez	2,700	Y	Coast	Guatemala	1948
142–48O.P.	Tiquisate	Escuintla	150	Y	Coast	Guatemala	1948
118A–46 #	Guatemala	Guatemala	4,855	W	Coast	Guatemala	1948
92–44O.P.	Retalhuleu	Retalhuleu	300	W	Coast	Guatemala	1948
129A–46 #	.....	.....	.....	Y	Coast	Venezuela	1948
12A–46 #⊗	Chocolá	Suchitepéquez	2,700	Y	Coast	Guatemala	1948
TGY	Tiquisate	Escuintla	150	Y	Coast	Guatemala	1949
1626–45O.P.	Chocolá	Suchitepéquez	2,700	W	Coast	Guatemala	1949
7A (WF9x38–11)	Antigua	Sacatepéquez	4,953	Y	Hybrid	Guatemala	1948
26A–46⊗	Sanarate	Progreso	2,632	W	Early Dwarf	Guatemala	1949
200–47O.P.	Patzún	Chimaltenango	7,000	W	Popcorn	Guatemala	1949

Key to Table 1:

O. P. Open pollinated commercial corn.
Sibbed pollinated within the variety.
⊗ Plants selfed within the variety and seed composited using 2 to 12 ears.
2s Plants of the seed selfed once, selfed the second time.
#⊗ Plants sibbed pollinated and selfed.
W Grain White.
Y Grain Yellow.

10A–46. Both of these selections are now in wide use on the Pacific coastal plain of Guatemala. The hybrid was made on the U. S. single cross WF9x38–11. It is thus half Guatemalan and half U. S. in origin. The last entry (200–47 O.P.) in the table was a large late popcorn belonging to the giganteum group *(20)*.

Other data relating to these varieties are given in Table 1, although more detailed description will be published elsewhere *(15)*. All of the seed analyzed was obtained from plants grown in Antigua, Guatemala, from the seed collected as indicated. From the time collected (Table 1) until analysis, they were stored in small sacks on open shelves in an inside room. Five samples of one variety grown during the same growing season in 5 different localities are also included in the study.

Moisture, crude fiber, ether extract, ash, and calcium were determined by A.O.A.C. official methods *(1)*. The digestion for nitrogen determination was done by the official method *(1)* and the distillation and titration as recommended by Hamilton and Simpson *(10)*. Iron was determined by the alpha, alpha' dipyridyl method of Hill *(11)* as modified by Jackson *(16)* and Moss *(22)*. The whole corn sample was ground approximately 15 mesh in a Hobart Mill before weighing except for mineral assays. In the latter case a porcelain mortar and pestle were used. Correlation coefficients and standard deviations were computed as suggested by Snedecor *(26)*.

RESULTS

The results are given in Table 2. Certain varieties are distinctly superior to others in their total nitrogen content with a range of 1.09 to 1.92 and an average value of 1.52% ($s = 0.22$). Using a conversion factor of 6.25 this represents a range in protein content of the corns of 6.8 to 12.0%. There was a difference in the average nitrogen

TABLE 2

Fat, crude fiber, nitrogen, ash, phosphorus and iron content in twenty-four varieties of corns [a]

Variety no.	Fat	Crude fiber	Nitrogen	Ash	Phosphorus	Iron
	%	%	%	%	*mg./100 gm.*	*mg./100 gm.*
206-44 ⊗	5.54	1.3	1.64	1.85	424	5.14
159–44 # (2s)	6.80	1.2	1.31	1.62	368	3.46
47A–46 O.P.	4.95	1.3	1.69	1.70	345	2.36
1470–46 #	5.74	1.7	1.57	.....	.....	.....
25A–46 ⊗	5.95	1.2	1.49	1.54	364	3.26
92A–46 # ⊗	6.86	1.5	1.29	1.47	343	2.83
192–44 # ⊗	5.65	1.2	1.70	1.41	277	2.61
15A–46 O.P.	4.82	1.4	1.12	1.37	313	2.76
166–44 O.P.	7.32	1.5	1.65	1.44	336	3.54
31–44 #	7.62	1.4	1.20	1.33	303	1.98
20–47 ⊗	5.61	1.1	1.09	1.08	249	2.86
1483–45 ⊗	6.25	1.6	1.21	1.43	311	2.07
21A–46B #	3.80	1.2	1.66	1.60	342	2.99
10A–46 O.P.	4.71	1.5	1.57	1.55	338	2.40
142–48 O.P.	5.02	1.4	1.66	1.42	212	2.08
118A–46 #	5.44	1.4	1.22	1.50	303	2.52
92–44 O.P.	5.00	1.4	1.78	1.55	374	4.96
129A–46 #	4.60	1.8	1.51	1.39	257	2.52
12A–46 # ⊗	6.07	1.7	1.65	1.39	333	2.50
TGY	4.55	1.4	1.54	1.47	222	2.44
1626–45 O.P.	4.48	1.5	1.57	1.51	367	2.96
7A (WF9x38–11)	4.88	1.7	1.61	1.43	226	5.32
26A–46 ⊗	6.36	1.4	1.79	1.50	339	2.56
200–47 O.P.	7.23	1.0	1.92	1.57	401	2.87

Key to Table 2 same as Table 1.

[a] All values expressed at 10% moisture and are the average of two determinations.

of corns grown in 1948 and 1949 with the average value of 1.57% for 1948 being higher than that of 1.44% for the following year.

The ether extractable portion of the corns ranged from 3.80 to 7.62 with an average value of 5.63 ($s = 0.97$). The crude fiber content ranged from 1.0 to 1.8 with an average of 1.40% ($s = 0.21$). The values for fat and crude fiber showed no clear difference in range or average among corns grown in 1948 and 1949. The iron content ranged from 1.98 to 5.32 with an average value of 3.00 mg. per 100 g. ($s = 0.92$). The readiness with which samples for iron assay become contaminated with traces of this element probably accounts for some of the high values and much of the variation. Phosphorus was also determined by the method of Fiske and Subbarow *(7)* as modified by Lowry and Lopez *(19)* and a range of 212 to 424 was obtained. The average phosphorus content was 320 mg. per 100 g. ($s = 58$). Calcium cannot be reported due to the use of a calcium containing dust to protect the samples from insect attack.

The yellow and white corns raised in 1948 and 1949 were compared separately for all the nutriments studied. No consistent differences could be found in nitrogen, crude fiber, and ether extract but, in both 1948 and 1949, the white corns were higher than the yellow corns in phosphorus and iron. Omitting the popcorn (200–47 O.P.) there was a tendency for a negative correlation ($r = -0.57$, 1948 and $r = -0.38$, 1949) between altitude of origin and nitrogen and a positive correlation ($r = +0.56$, 1948 and $r = +0.53$, 1949) between fat and altitude of origin in both years. Thus the nitrogen content appears higher in the varieties originating in the lowlands than in those from the highlands, although none of the correlations are statistically significant at the 5% level. The content of fat tended to be higher in the highland varieties. The crude fiber, ash, and mineral contents showed no such trends.

In order to obtain data relating to the effect of environmental differences on the content of the nutrients discussed, samples of one variety (TGY) were analyzed from 5 localities in the spring growing season of 1951. The results are reported in Table 3

TABLE 3

Fat, crude fiber, nitrogen, ash, phosphorus and iron of variety TGY grown at 5 localities [a]

Origin	Altitude	Fat	Crude fiber	Nitrogen	Ash	Phosphorus	Iron
	feet	%	%	%	%	*mg./100 gm.*	*mg./100 gm.*
Tiquisate	150	5.18	1.8	1.41	1.39	344	2.62
Cuyuta	100	4.60	1.8	1.37	1.43	354	2.49
Ratalhuleu	200	4.74	1.9	1.50	1.28	316	2.02
Coatepeque	100	5.19	1.3	1.43	1.18	296	1.84
Antigua	4,953	4.30	1.3	1.52	1.18	284	2.80

[a] All values expressed at 10% moisture and are average of two determinations.

and show a relatively small variation in nitrogen content, 1.37 to 1.52%, despite an altitude range of 100 to 4,953 feet in the localities in which they were grown. The variations in fat, crude fiber, ash, and iron are correspondingly small. The variation in phosphorus, shown in the table, 284 to 354 mg. per 100 g., was also smaller than that among the varieties listed in Table 1.

DISCUSSION

For a population almost wholly dependent on corn for their dietary protein, the differences in protein content reported have considerable practical nutritional significance. The protein contribution of corn to the diet of persons consuming 500 g. of it, a common intake in the Guatemalan highlands, would vary from 34 to 56 g. according to the results of Table 1. All of these corns, except two, as grown in their original localities were eaten by large numbers of people and considered good for their area. By contrast a corn sample selected from Santa María Cauqué and in common use

in this village had a protein content equivalent of only 29 g. in 500 g. of corn. Of course, the nutritional value of these corns is dependent upon the quality of the protein as well as its quantity, but qualitative differences could not wholly mask the nutritional effect of quantitative differences of this magnitude. Data relating to the quality of protein in these corns will be discussed in the next two papers of this series.

Differences in the nutritive value of these corns due to variations in the other nutriments listed also occur but are over-shadowed from the point of view of human nutrition by the relatively large differences in protein content. The corns with higher ether extractable portions are markedly higher in caloric value. Since the Central American diets tend to be extremely low in fat, and the fat content of corn often constitutes the bulk of the fat in the diet, these variations may have direct nutritional significance. The differences in ether extractable portion are also of commercial significance to corn milling industries.

The question arises as to what proportion of these differences in composition are due to inherent genetic factors and what proportions to varying cultural and environmental influences. Since the corns were all grown in the same locality the effect of environment should have been minimized. This is true within a single growing season, but all of these corns were not grown in the same year and small differences in rainfall, temperature, and amount of sunshine occurred from year to year. Due to the variations among varieties grown in the same year, it can be stated that a considerable degree of genetic variation in the nutrients studied did occur.

It likewise cannot be expected that these corns would necessarily have the same relative standing if grown in another locality or even the same locality in a year with markedly different climatic conditions *(20)*. Corn is notoriously best adapted to specific environments, and frequently the best corn in a given region does poorly when transferred to a different one *(17, 18)*. The tendency toward a negative correlation of protein content with altitude of origin, although not statistically significant, is suggestive of the relationship of nutrient content to the environment to which the strain is adapted. The tendency of ether extract to be positively correlated with altitude merits comment. Highland corns have a longer maturity period. Although no local data are at present available, it is possible that accumulation of fat may take place in larger amounts than in corns of the lowlands which have a period of maturity of around 100 days. Future studies will be designed to determine the relation of nutritive value to altitude but it is obvious that yield and maturity *(13)* data will be indispensable in the evaluation of the agricultural significance of the phenomena reported or indeed in any studies of the nutritive value of food crops.

The agricultural as well as nutritional significance of the variation in nutritive value of corns reported will be greatly reinforced if the results of Table 3 are borne out by further studies. Relatively wide differences in altitude and other environmental conditions resulted in very small differences in nitrogen content compared with those reported for Table 2. The corn selected was not as well adapted and did not grow as well in the highlands as in the coastal area for which it was developed, yet genetic constitution apparently had a much greater influence on nitrogen content

than the considerable environmental differences. The data clearly suggest that the search for and the development and distribution of varieties of corn superior in both yield and nutritive value hold great promise and should be continued, especially in the Central American area where corn is an important human food and shows great genetic variation.

SUMMARY

Twenty-three strains of corn of Guatemalan, Mexican, or Venezuelan origin grown at 5,000 feet in Antigua, Guatemala, together with one Guatemalan-United States hybrid cross were analyzed for moisture, nitrogen, ether extract, crude fiber, iron, and phosphorus. All values were adjusted to an average moisture of 10%. The nitrogen averaged 1.52% ($s = 0.22$), the ether extract 5.63% ($s = 0.97$), crude fiber 1.40% ($s = 0.21$), iron 3.00 mg. per 100 g. ($s = 0.92$) and phosphorus 320 mg. per 100 g. ($s = 58$). All were grown at an intermediate altitude, but there is a tendency for corns from seeds collected at low altitudes to have higher nitrogen and lower fat contents than those obtained from high areas as illustrated by the correlations ($r = -0.57$, $r = +0.56$ in 1948) and ($r = -0.38$, $r = +0.53$ in 1949).

One variety was grown in 5 different locations during the spring growing season of 1951 with relatively little variation in nitrogen content (1.37 to 1.52%) and in the other nutrients measured. It is concluded that although environment also influences nutritional value, important genetic differences in nutritive composition, especially in nitrogen and ether extract, do exist among Guatemalean corns and can be used in the selection of varieties for improved nutritional value.

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THE NUTRITIVE VALUE OF CENTRAL AMERICAN CORNS. II. LYSINE AND METHIONINE CONTENT OF TWENTY-THREE VARIETIES IN GUATEMALA[a]

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On a dietary regimen in which the protein requirement is supplied only by corn, unsatisfactory growth occurs in animals *(9)* due to insufficient quantities of one or more of the essential amino acids. Zein, one of the principal proteins of corn, is deficient in lysine and tryptophane *(10)*. The remaining corn proteins, although containing appreciable amounts of tryptophane and lysine, are not able to compensate sufficiently to permit maximum animal growth. The relative proportions of zein and other proteins vary according to the nitrogen content of the corn *(14)*. Since marked variation in nitrogen content has been shown for several Central American corns *(1)*, variations in essential amino acids may also be expected.

In areas such as Central America where corn supplies 80% of protein in the diets of some rural families, the selection of corns for high protein quality through study of their genetic and environmental variation in amino acid content has great potential nutritional and agricultural importance. The data presented are intended to aid in a corn improvement program for the region as well as furnish information concerning variations in the nutritive value of corns.

In this study twenty-three of twenty-four corn varieties previously analyzed for moisture, nitrogen, crude fiber, fat, ash, calcium, and iron *(1)* together with samples of one of these varieties grown in five different localities have been analyzed for their lysine and methionine content.

MATERIALS AND METHODS

Twenty-two varieties of corn and a cross between a Guatemalan and United States commercial hybrid were planted in one locality, Antigua, Guatemala, during 1947, 1948, and 1949. The characteristics and origin of these corns have been described previously *(1)*. Representative samples of seed harvested from these plantings, and five samples obtained from one of the varieties, which was grown in five different localities during the same season, were assayed for lysine and methionine. The samples were analyzed by the microbiological methods of Horn *et al. (7, 8)*. Each sample was assayed in dupli-

[a]A cooperative study in which the corn samples and data relating to their origin were furnished by Dr. Irving E. Melhus, Director, Iowa State College Tropical Research Center, Antigua, Guatemala. The project was aided by a research grant from the Instituto de Fomento de la Producción (INFOP) Guatemala, Central America. Contribution I-23 from the Instituto de Nutrición de Centro América y Panama.

cate at four concentrations and the entire procedure of hydrolysis and analysis was repeated if the results were not consistent.

RESULTS

The values obtained are presented in Table 1 and expressed both in terms of 10% moisture content and on the basis of 16% nitrogen. It will be seen that on a 10% moisture basis the methionine content varied from 0.10% to 0.22% in the popcorn. The highest methionine value for field corn was 0.18%. The average value for methionine in twenty-two field corns, omitting the popcorn, was 0.14% ($s = 0.02$). The lysine con-

TABLE 1

Methionine and lysine content of corns

Corn	Calculated on the basis of 10% moisture		Calculated on the basis of 16% nitrogen		Percent of the requirement supplied by 500 g. of corn	
	Methionine	Lysine	Methionine	Lysine	Lysine	Methionine
	%	%	%	%		
206–44⊗	0.15	0.37	1.46	3.60	231	68
159–44 # (2s)	0.16	0.40	1.95	4.87	250	72
47A–46O.P.	0.15	0.30	1.42	2.84	157	68
15A–46O.P.	0.11	0.30	1.57	4.29	188	50
166–44O.P.	0.13	0.30	1.26	2.91	187	59
1470–45 #	0.15	0.37	2.53	3.77	231	68
31–44 #	0.18	0.28	2.41	3.75	175	80
20–47⊗	0.16	0.28	1.37	4.16	175	72
1483–45⊗	0.10	0.29	1.32	3.84	181	45
25A–46⊗	0.12	0.28	1.29	3.00	175	54
92A–46 #⊗	0.17	0.37	2.11	4.60	231	77
192–44 #⊗	0.10	0.29	0.94	2.74	237	59
TGY	0.15	0.29	1.56	3.02	175	67
10A–46O.P.	0.16	0.35	1.63	3.56	218	72
118A–46 #	0.12	0.34	1.58	4.48	212	54
1626–45O.P.	0.11	0.36	1.12	3.66	225	50
92–44O.P.	0.15	0.34	1.34	3.05	212	68
129A–46 #	0.16	0.31	1.69	3.28	193	72
21A–46B #	0.14	0.29	1.35	2.80	181	63
12A–46 #⊗	0.12	0.33	1.16	3.20	206	54
26A–46⊗	0.12	0.05	1.07	0.44	31	54
7A(WF9x38–11)	0.11	0.31	1.09	3.08	193	50
200–47O.P.	0.22	0.30	1.86	2.50	187	100

Key to Table 1:

O. P. Open pollinated commercial corn.
\# Sibbed pollinated within the variety.
⊗ Plants selfed within the variety and seed composited using 2 to 12 ears.
2s Plants of the seed selfed once, selfed the second time.
#⊗ Plants sibbed pollinated and selfed.
W Grain White.
Y Grain Yellow.

tent varied from 0.28 to 0.40% except for one extraordinarily low value of 0.05%. The complete hydrolysis and assay procedure, repeated 4 times on this sample, gave 0.06, 0.04, 0.07, 0.05%. The average value for lysine in 22 varieties (omitting 142–48) was 0.32% ($s = 0.04$). When they are calculated on the basis of 16% nitrogen, the average values are 1.53% and 3.50% for methionine and lysine respectively. The standard deviations of 0.39 and 0.65 reflect the considerable variation among varieties.

There is no apparent correlation between the amount of nitrogen per cent reported previously *(1)* and the content of methionine ($r = 0.110$) and lysine ($r = 0.114$) in these corns. Data presented in Table 1 also show the percentage of the suggested minimum requirement of lysine and methionine for adult maintenance *(12)* supplied by 500 g. of whole corn. The quantity of corn was selected for the comparison, because it represents

the approximate amount consumed per person per day in many indigenous populations of rural Guatemala.

All of the corns described above were raised in one locality. It can be seen from Table 2 that the range of variation is considerable for a single corn variety grown in different localities, being from 0.12 to 0.22% with an average of 0.15 ($s = 0.04$) for methionine and 0.38 to 0.45% with an average of 0.40 ($s = 0.05$) for lysine.

TABLE 2

Lysine and methionine content of corn variety TGY raised in five different environmental situations

No.	Locality	Altitude in feet	Grams percent			
			10% Moisture		16% Nitrogen	
			Methionine	Lysine	Methionine	Lysine
271	Coatepeque	100	0.12	0.44	1.35	4.93
275	Cuyuta	100	0.13	0.45	1.49	5.16
266	Tiquisate	150	0.12	0.38	1.41	0.70
298	Retalhuleu	200	0.16	0.34	1.70	3.58
276	Antigua	4,953	0.22	0.41	2.26	4.30

DISCUSSION

It is obvious from the data presented that significant differences in the content of the essential amino acids lysine and methionine do occur in corn. Previous studies have shown that other amino acids vary in a similar manner *(2, 4)*. These variations in amino acid content are often independent of the nitrogen variations *(1, 2, 5)*, and may be either environmental, genetic, or both, depending on the material studied *(2, 3, 4, 5, 6, 16, 17)*.

Although, with one exception, the variations in Table 1 represent two different years, the differences among the varieties, within each year, are considerable. These differences can be considered primarily genetic and confirm the findings of other workers *(2, 5)* that amino acids in corn protein are affected by the genetic constitution of the plant. Table 2 illustrates the influence of environmental factors on lysine and methionine content.

The lysine supplied by 500 g. of corn seems to be approximately twice the minimum requirement for adult human maintenance. This means that in the diet of many adults in Guatemala, corn alone supplies approximately the recommended daily allowance of lysine *(12)*. Obviously the adequacy of the amino acid content of diets for families and populations cannot be determined by chemical or microbiological analysis until the requirements for growth as well as maintenance are known.

The high replaceability of methionine by cystine for human maintenance was reported after the analytical work had been completed *(13)*. Unfortunately, cystine values are not available to permit any estimate as to the total methionine activity of these corns. However, it is clear from the data of Table 1 that the methionine alone in 500 g. of the corns studied is insufficient to supply the minimum requirement for this amino acid.

The extraordinarily low lysine content of corn 26A-46 cannot be ignored. A precedent for such a variation lies in the studies of Richey and Dawson *(11)* and Teas and Newton *(15)* with the tryptophane and niacin content of x-ray induced mutants. In these studies variations of over 400% were observed between some induced mutation and the strain from which they

originated. Whether a spontaneous biochemical mutation has been accidentally encountered can be determined only by further study of this variety.

From a practical point of view a variety of corn cannot be introduced into a region for its higher nutritive value alone. It must have a higher yield or other desirable characteristic obvious to the farmer. Most of the varieties in Table I were selected because they were corns that grew unusually well in their region. The best of these for the Antigua area should be increased and distributed as soon as possible to this limited region.

Since corns are notoriously adapted to a limited range of conditions *(3, 4, 6, 16, 17)*, the best corn from the standpoint of nutritive value or yield as grown in the Antigua area may do poorly in one or both of these characteristics if grown under another set of conditions. However, the results presented are encouraging in that they do indicate marked genetic variation in the methionine and lysine content of corn that the plant breeder should be able to use to advantage in his overall efforts to develop improved corn varieties.

SUMMARY

Twenty-three varieties of Guatemalan field corn grown in one locality were analyzed for their methionine and lysine contents by microbiological methods. The average value was 0.14% (0.10 to 0.22) for methionine and 0.32% (0.28 to 0.40) for lysine.

One of these corns grown in 5 widely different localities ranged from 0.12 to 0.22% in methionine and from 0.06 to 0.45% in lysine with an average of 0.15 and 0.34, respectively, variations associated with environmental factors. Values are calculated on the basis of 10% moisture.

One of these corns not included in the lysine average had an unaccountably low lysine content of 0.05%. A popcorn had 0.22% methionine and 0.30% lysine, compared with 0.11 and 0.31% for a cross between a Guatemalan field corn and a U. S. hybrid. The percentage of the minimum requirement for adult human maintenance supplied by 500 g. of the field corns studied range from 175 to 250 for lysine. The methionine alone in this quantity supplied from 45 to 80% of the minimum methionine activity required. The availability of cystine to meet part of the methionine requirement was not determined.

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THE NUTRITIVE VALUE OF CENTRAL AMERICAN CORNS. III. TRYPTOPHANE, NIACIN, THIAMINE, AND RIBOFLAVIN CONTENT OF TWENTY-THREE VARIETIES IN GUATEMALA[a]

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Despite the frequent occurrence elsewhere of pellagra attributed to niacin deficiency in persons consuming high corn diet *(3)*, clinical surveys in rural Central America have not revealed a significant incidence of this deficiency disease. Since corn is a source of 80% of the niacin in some Guatemalan families *(17)*, determinations of the niacin content of Guatemalan corns are of special interest. Tryptophane can be converted to niacin *(8, 15)* and a deficiency of niacin in the diet can be corrected by tryptophane, so the determination of this amino acid is essential to an understanding of the incidence of niacin deficiency.

Apart from its relation to the metabolism and requirement of niacin, tryptophane is one of the essential amino acids, which may limit human growth on predominantly vegetable diets. Any research program concerned with the protein quality of corn must include tryptophane analysis, especially, since zein, one of the principal proteins of corn, has been shown to be deficient in tryptophane *(21)*, at least for rat growth.

Teas and Newton *(31)* have described great variations in the content of tryptophane, niacin, and indoleacetic acid between mutant and normal strains of corn which could not be explained as due to simple arrest of development, differences in size of kernels, or to storage. Teas *et al. (32)* have also reported differences between sugary and starchy corn kernels arising late in development. Richey and Dawson *(26)* state that the niacin concentration in corn is a function of the genetic constitution of the endosperm with practically no influence of kernel size or environment. Frey *(9)* found no correlation of tryptophane with total nitrogen content.

There are few published data on the effect of genetic and environmental factors on the thiamine and riboflavin content of corn. Jaffé, Budowski, and Gorra *(18)* determined the thiamine, riboflavin, and niacin content of 17 varieties of Venezuelan corn and reported relatively small fluctuations

[a]A cooperative study in which the corn samples and data relating to their origin were furnished by Dr. Irving E. Melhus, Director, Iowa State College Tropical Research Center, Antigua, Guatemala. The project was aided by a research grant from the Instituto de Fomento de la Producción (INFOP) Guatemala, Central America. Contribution I-24 from the Instituto de Nutrición de Centro América y Panamá.

among the corns studied. Hunt *et al. (16)* studied the environmental and agronomic factors influencing the B vitamin content of corn, wheat, and oats, and reported that additions of phosphorus, potassium, and nitrogen to unlimed soil increased the percentage of thiamine in corn kernels in 2 out of 3 years and decreased the niacin each year of the 3-year experiment. The same fertilizers applied to limed soil increased the thiamine in corn kernels in 2 out of 3 years and increased the nicotinic acid content once in 3 years.

The proportion of the diet represented by corn is so large in some Guatemalan Indian families that 85% of the thiamine and 50% of the riboflavin are calculated to come from this source. The marked variations in nitrogen, methionine, and lysine have already been reported for the corns studied *(1, 4)*. Similar significant variations for tryptophane, niacin, thiamine, and riboflavin are described in this paper.

MATERIALS AND METHODS

Twenty-two varieties of corn of different origins and a cross between a Guatemalan and a United States commercial hybrid were planted in one locality, Antigua, Guatemala, during 1947, 1948, and 1949. The characteristics and origin of these corns were described previously *(4)*. Representative samples of seed harvested from these plantings and 5 samples obtained from one of the varieties grown in 5 different localities during the same season were assayed for tryptophane, niacin, riboflavin, and thiamine.

Tryptophane was estimated by the microbiological method of Wooley and Sebrell *(34)* with the modification that prior to the enzymatic hydrolysis the material was autoclaved at 15 pounds' pressure with 75 ml. of water for 30 minutes and the assay media were prepared as suggested by Greene and Black *(12)*. Niacin was determined microbiologically *(25)*. *Lactobacillus arabinosus* 17–5 was used in both assays. Riboflavin was determined by the fluorometric method of Hodson and Norris *(14)* and thiamine by the thiochrome method of Hennessey and Cerecedo *(13)*. Analyses of variance were carried out as described by Snedecor *(30)*.

RESULTS

The tryptophane and niacin values are presented in Table 1. When adjusted to a a common 10% moisture value, the tryptophane content varied from 0.025% to 0.059% with an average of 0.046 ($s = 0.009$). Although the negative sign of the correlation coefficient is suggestive, there was no significant correlation under the conditions described between the levels of tryptophane and those previously reported for nitrogen in the same varieties ($r = -0.443$).[b] The variation in niacin is also large, 1.25 to 2.67 mg. per 100 g., with an average of 1.77 ($s = 0.35$). There was no significant correlation between the values for niacin and those for nitrogen *(1)* or tryptophane.

In the last two columns of Table 1, the percentages of minimum adult requirements for tryptophane and niacin supplied by the amount of each of these nutrients in 500 g. of whole corn of the varieties are listed. This quantity of corn is used in the comparison because it represents the approximate amount consumed per person per day by adults in many Indian communities in rural Guatemala *(17)*. Seven of the corns would supply 100% or more and seven 90 to 99% of the minimum daily requirement of 0.25 g. of tryptophane recommended by Rose *(27)*. The average value was 88% with a range of 50 to 118%. Only two of the corns supplied 75% of the daily allowance of 15 mg. of niacin recommended by the National Research Council (U.S.). The values range from 42 to 89% with an average of 59%.

One of these varieties (TGY), a widely used commercial yellow corn, was grown in five different localities during 1949 and samples from each locality analyzed with the results given in Table 2. It will be seen that the range of variation is considerable,

[b] This accounts for the greater range of variation, 0.28 to 0.84%, with an average of 0.50 ($s = 0.15$) when calculated on a basis of 16% nitrogen.

TABLE 1

Niacin and tryptophane content of corns

Corn	Calculated on the basis of 10% moisture		Calculated on the basis of 16% nitrogen	Percent supplied by 500 grams of whole corn	
	Niacin	Tryptophane	Tryptophane	Requirement Tryptophane[c]	Allowance Niacin[d]
	mg./100 gm.	%	%	%	%
206–44 ⊗	1.69	0.049	0.48	98	56
159–44 # (2s)	1.69	0.053	0.65	106	56
47A–46 O. P.	1.46	0.048	0.45	96	49
15A–46 O. P.	1.68	0.059	0.84	118	56
166–44 O. P.	2.67	0.039	0.38	78	89
1470–45 #	1.39	0.058	0.59	116	46
31–44 #	1.58	0.056	0.75	112	53
20–47 ⊗	1.93	0.057	0.87	114	64
1483–45 ⊗	1.70	0.040	0.53	80	57
25A–46 ⊗	1.68	0.035	0.38	70	56
92A–46 # ⊗	2.17	0.039	0.48	78	72
192–44 # ⊗	1.74	0.030	0.28	60	58
TGY	1.25	0.049	0.51	98	42
10A–46 O. P.	1.51	0.048	0.49	94	51
118A–46 #	1.87	0.048	0.63	96	62
1626–45 O. P.	1.59	0.025	0.25	50	53
92–44 O. P.	2.50	0.039	0.35	78	83
129A–46 #	1.97	0.046	0.49	90	66
21A–46B #	1.96	0.034	0.33	68	66
12A–46 # ⊗	1.45	0.037	0.36	74	48
26A–46 ⊗	2.09	0.050	0.47	100	70
7A (WF9x38–11)	1.29	0.059	0.59	118	43
200 47 O. P.	1.76	0.049	0.41	98	59

Key to Table 1:

O. P. Open pollinated commercial corn.
\# Sibbed pollinated within the variety.
⊗ Plants selfed within the variety and seed composited using 2 to 12 ears.
2s Plants of the seed selfed once, selfed the second time.
#⊗ Plants sibbed pollinated and selfed.
W Grain White.
Y Grain Yellow.

[c] A value of 0.25 was taken as the human tryptophane requirement for adult maintenance (*27*).
[d] The National Research Council (U. S) recommended daily allowance for niacin.

TABLE 2

Tryptophane, niacin, thiamine and riboflavin content of corn variety TGY raised in five different environmental situations

No.	Locality	Altitude in feet	Calculated on the basis of 16% nitrogen tryptophane	Calculated on the basis of 10% moisture			
				Trypto-phane	Niacin	Thiamine	Riboflavin
			%	%	*mg./100 gm.*	*mg./100 gm.*	*mg./100 gm.*
271	Coatepeque	100	0.34	0.030	2.12	0.41	0.15
275	Cuyuta	100	0.40	0.034	1.55	0.66	0.14
266	Tiquisate	150	0.67	0.059	1.73	0.55	0.11
298	Retalhuleu	200	0.14	0.013	1.88	0.66	0.14
276	Antigua	4,953	0.37	0.035	1.56	0.69	0.12

from 0.013 to 0.059% for tryptophane and from 1.55 to 2.12 mg. per 100 g. for niacin. The average values are 0.034 ($s = 0.016$) for tryptophane and 1.77 ($s = 0.24$) for niacin. The variation in thiamine and riboflavin in the corn TGY grown at different localities is given in Table 2. The thiamine content ranged from 0.41 mg. per 100 g. to 0.69 mg. per 100 g. with an average of 0.59 ($s = 0.12$). The riboflavin content varied from 0.11 mg. per 100 g. to 0.15 mg. per 100 g. with an average of 0.13 ($s = 0.016$). The thiamine and riboflavin contents of the 23 varieties are presented in Table 3. On the basis of 10% moisture, the thiamine content varied from 0.71 mg. per 100 g. to 0.37 mg. per 100 g. with an average of 0.52 ($s = 0.029$); riboflavin ranged from 0.05 mg. per 100 g. to 0.11 mg. per 100 g. with an average of 0.08 ($s = 0.118$).

TABLE 3

Thiamine and riboflavin content of twenty-three varieties of corn*

Corn	Calculated on the basis of 10% moisture		Corn	Calculated on the basis of 10% moisture	
	Thiamine	Riboflavin		Thiamine	Riboflavin
	mg./100 gm.	*mg./100 gm.*		*mg./100 gm.*	*mg./100 gm.*
206–44 ⊘	0.51	0.08	TGY	0.45	0.10
159–44 # (28)	0.44	0.08	10A–46 O. P.	0.42	0.10
47A–46 O. P.	0.70	0.07	118A–46 #	0.57	0.09
15A–46 O. P.	0.61	0.05	1626–45 O. P.	0.56	0.07
166–44 O. P.	0.58	0.10	92–44 O. P.	0.46	0.06
1470–45 #	0.56	0.08	129A–46 #	0.52	0.11
31–44 #	0.40	0.09	21A–46B #	0.54	0.08
20–47 ⊘	0.39	0.06	12A–46 # ⊘	0.49	0.08
1483–45 ⊘	0.37	0.05	26A–46⊘	0.50	0.07
25A–46 ⊘	0.53	0.09	7A (WF9x38–11)	0.39	0.09
92A–46 # ⊘	0.52	0.07	200-47 O. P.	0.71	0.06
192-44 # ⊘	0.58	0.09			

*The mean and standard deviations were calculated using the values of the varieties listed in paper I of this series *(1)*.

DISCUSSION

The data presented show that although the range of values is considerable, some of the corns studied have sufficient tryptophane in the quantities consumed to meet the minimum estimated requirement for adult human maintenance.

The values cited also indicate that the corn alone in some Guatemalan communities supplies an average of 73% of the National Research Council (U. S.) recommended allowance for niacin. The niacin intake can be somewhat lower than this without the appearance of clinical symptoms of pellagra *(10, 11)*. Furthermore, recent work *(5, 19, 23)* suggests that normally unavailable niacin precursors in corn are made available by lime treatment to which corn is subjected in Guatemala before tortilla preparation.

When the total niacin from all sources was estimated by dietary survey, it was found to average slightly more than 14 mg. daily per adult in 5 Guatemalan villages studied *(17)*. The true niacin equivalent of these diets is probably somewhat higher due to the conversion of some tryptophane to niacin and possibly the availability of niacin precursors in tortillas. Such a diet should not be pellagra producing unless a specific niacin antagonist or pellagra producing factor is present in corn. A great deal has

been written about the relation between a corn diet and pellagra *(2, 6, 7, 20, 21, 22, 28, 29, 33, 36)*, but the existence of a specific pellagra promoting factor in corn is still not proved.

Diets high in corn which have been reported to be pellagra producing usually have contained an abundant source of calories but did not supply sufficient protein, as for example, the fat, corn, and molasses diets which formerly were typical of pellagra areas in the southern United States. The high corn diet of the populations of the Guatemalan highlands furnishes a higher percentage of both protein and calories from corn than is provided by the so-called classical pellagra producing corn diets. It seems likely that the problem will be reduced to one of the amount of niacin activity and the quantity of tryptophane in the total diet in excess of that required for protein synthesis.

It has been reported that the niacin concentration of corn is practically uninfluenced by kernel size or environment *(26, 31)*. While the present data justify the conclusion that major genetic differences occur, it is difficult to explain the variation in niacin content in the same corn grown in five different localities (Table 2) without postulating the influence of environmental factors. This conclusion is in agreement with Hunt *et al. (16)*.

SUMMARY

Twenty-three varieties of Guatemalan field corn were analyzed for their tryptophane and niacin contents by microbiological methods and thiamine and riboflavin by chemical methods. The average values were 0.046% (0.025 to 0.049) for tryptophane, 1.76 mg. per 100 g. (1.25 to 2.67) for niacin, 0.52 mg. per 100 g. (0.37 to 0.71) for thiamine, and 0.08 mg. per 100 g. (0.05 to 0.11) for riboflavin. Although this variation was largely due to genetic factors, samples from one variety grown in five different localities show considerable variation ranging from 0.013 to 0.059 for tryptophane, from 1.56 to 2.12 for niacin, from 0.41 to 0.69 for thiamine, and from 0.11 to 0.15 for riboflavin, differences which must be attributed largely to environmental factors.

The average daily consumption of corn per adult in some areas of Guatemala is as high as 500 g. On the basis of a minimum requirement of 0.25 mg. of tryptophane for adult human maintenance, this quantity of the corns studied provided from 50 to 118% of tryptophane needed. It also supplied 42 to 89% of the National Research Council (U. S.) recommended allowance for niacin and an apparently adequate intake of thiamine.

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A NEW V-8 MEDIUM FOR DETERMINING LACTOBACILLI[a]

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Many media have been suggested for the isolation and enumeration of lactic acid bacteria. It has been felt that the media now in use have not been specific enough for the detection of these organisms since they allow other microorganisms to grow abundantly, completely overgrowing the lactic acid bacteria in a short time. Some media will support growth of the lactic acid bacteria, but the colonies are often small and difficult to count. After working with the many different media used for this type of work, experiments with a new medium were started in 1949. The objective was to develop a medium which would grow the acid-producing bacteria quickly and to a larger size than any of the media commonly used for this group of bacteria. Another specification for a new medium was that it permit the acid-producing bacteria to grow to the exclusion of many other types of bacteria commonly found in association with the acid-producing bacteria. These other bacteria frequently overgrow the acid-producing type and hence it is not possible to obtain an accurate plate count nor to isolate them.

REVIEW OF THE LITERATURE

Because of the importance of studying the lactic acid bacteria, many investigators have attempted the preparation of nutrient media for cultivating these organisms.

In 1920, Ayers and Mudge *(1)* prepared a milk-powder medium for the determination of bacteria in milk. This medium gave the total count and also enumerated the strong and weak acid-producers, the alkali-forming, the inert, and the peptonizing bacteria.

Fabian, Bryan, and Etchells *(9)* found the milk-powder agar of Ayers and Mudge *(1)* to be an excellent medium for growing the bacteria found in fermenting pickle brine and also that it differentiated the bacteria into the 4 main groups mentioned.

Rettger and Kulp *(20)* tried to find a substitute for whey broth and whey agar, because these required an exacting technique to obtain a clear medium. The same authors in a later paper reported the casein and Klim digest media to be superior for cultivating *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* and that galactose appeared to be the most favorable carbohydrate *(15)*. Kulp *(14)* found that a tomato juice medium containing peptone, agar, and distilled water and adjusted to pH 7.0 gave comparative results with whey-galactose agar and a digest galactose medium and was more easily prepared.

In an attempt to isolate lactobacilli from soil and grain, Hunt and Rettger *(12)* had difficulty in using an acid enrichment broth containing a fermentable carbohydrate since fungi readily overgrew the lactobacilli.

Weiss and Rettger *(23)* used a tomato broth medium for isolating *Lactobacillus bifidus*.

Barber and Frazier *(2)*, in a study of the dissociants of lactobacilli, used a modification of the carrot liver agar described by Garey, Foster, and Frazier *(10)*. They used United States flake agar in place of washed agar and also an infusion of ground beef liver instead of the liver extract.

McLaughlin *(17)* reported a medium which gave much better results than the commercial whey and tomato juice agars. This medium was called "trypticase sugar agar"

[a]Journal article No. 1432, Michigan State Experiment Station, East Lansing, Mich.

and contained pancreatic digest of casein, lactose, glucose, sucrose, gelatin, and agar adjusted to pH 6.0.

Wade, Smiley, and Smith *(22)* suggested a method of obtaining the ratio of acid-forming to non-acid-forming bacteria. This medium, a modification of the one suggested by Garey, Rittschoff, Stone, and Boruff *(11)*, consisted of filtered tomato juice, yeast extract, glucose, and a buffer. To this was added $CaCO_3$ and an alcoholic solution of brom cresol purple.

A medium which reduced the number of common contaminants found when isolating lactobacilli from oral, vaginal, and fecal samples was devised by Rogosa, Mitchell, and Wiseman *(21)*.

Emard and Vaughn *(6)* found that sorbic acid was effective in selectively favoring the growth of the catalase negative lactic acid bacteria.

In a study of the chemical and bacteriological changes in dill-pickle brines during fermentation, Jones, Veldhuis, and Veerhoff *(13)* used nutritive caseinate agar (Difco) for the bacterial analysis to which was added 0.4% from cresol purple as an indicator.

McCleskey, Faville, and Barnett *(16)*, in studying characteristics of *Leuconostoc mesenteroides* isolated from cane juice, employed a medium using raw sugar, tryptone, yeast extract, agar, and water and adjusted to pH 6.7.

The Committee on the Microbiological Examination of Foods *(4)* suggested a tryptone-glucose-yeast extract agar for the examination of sauerkraut.

Murdock, Folinazzo, and Troy *(18)*, in evaluating plating media for citrus concentrates, employed a number of different media to determine maximum counts of several leuconostoc, lactobacilli, and yeasts. They found that the orange serum agar at pH 5.4 was a suitable differential medium for the leuconostoc, lactobacilli, and yeasts. The orange serum agar consisted of tryptone, yeast extract, dextrose, K_2HPO_4, agar, orange serum, and distilled water.

Mrak, Phaff, and Douglas *(19)* used an infusion of four vegetables to obtain sporulation in yeasts. Henrici used V-8 juice to which was added sufficient agar to solidify it for the same purpose according to Wickerham *et al. (24)*. They *(24)* modified Henrici's medium by the addition of ½ of a yeast cake to 1 pint, 2 fluid ounces of V-8 juice which previously had been adjusted to pH 6.8 with KOH. This was steamed for 10 minutes, readjusted to pH 6.8 after which it was added to an equal volume of 4% melted agar and sterilized. This medium was suitable to obtain sporulation in yeasts.

From the review of literature it may be seen that vegetable juices in general and V-8[b] juice in particular has been used previously to isolate and study both yeasts and bacteria.

PRELIMINARY STUDIES

Six different media were prepared using V-8 vegetable juice as a base. The composition of the different media were as follows:

Medium	Tryptose	Glucose	Beef extract	Agar	V-8 Juice	pH
	g.	*g.*		*g.*	*ml.*	
A	10	5	3	18	500 unfiltered	4.6
B	10	5	3	18	250 filtered	5.1
C	10	5	....	18	250 filtered (adjusted)	6.1
D	10	5	....	18	125 filtered	5.6
E	10	5	3	18	500 filtered	4.5
F	10	5	3	18	500 filtered (adjusted)	5.8

These 6 media when tested with *Lactobacillus plantarum* showed media D and F to give the best results when compared with tomato juice agar. Since these media were to be used primarily as plating media for pickle and kraut fermentations, it was desirable to have an indicator present to distinguish the acid-producing bacteria and

[b] V-8 juice is a trade name used for a blend of 8 vegetable juices first manufactured by Standard Brands at Terre Haute, Indiana, but now manufactured by the Campbell Soup Co., Philadelphia, Pa.

to see how it affected sporogenic soil bacteria. Accordingly brom cresol green was chosen because its color range was from 3.8 to 5.4 and the pH of media D and F were near this range. Test organisms used were 2 cultures of *L. plantarum*, one from a pickle and the other from a kraut fermentation, and also the following species of the genus *Bacillus: cereus subtilis, mesentericus fuscus, vulgatus, megatherium*, and *pumilus*. Experiments using these 8 organisms and 0.005, 0.0075, 0.01, 0.02, 0.04, and 0.06 g., respectively, of B.C.G. per 100 ml. of media D and F showed 0.01 g. of the indicator gave the best results. Quantities less than 0.01 were not satisfactory due to lack of contrasting color while larger amounts had too great an inhibiting effect on the *L. plantarum*. These experiments also indicated that the 0.1% B.C.G. would inhibit the various species of *Bacillus* used which are commonly found in the soil.

Another series of tests were run with *L. plantarum* using media D and F with and without 0.1% B.C.G. indicator and another series in which lactose was substituted for dextrose. Thus in this series there was medium D with and without 0.1% B.C.G. in which dextrose was present and a duplicate set in which lactose replaced dextrose. A duplicate of this series was done also with V-8 medium F using tomato juice agar for comparison.

The results from this series of experiments showed that the presence of the indicator did not reduce materially the number or size of the colonies. They also showed that medium D with dextrose had more colonies per plate but that medium F with lactose had slightly fewer but much larger and more distinct colonies. The colonies on medium F were 3 to 4 times larger than those in tomato juice agar.

To determine the optimum pH of the V-8 media D and F a series of experiments was run at pH's 6.55, 6.18, 5.79, 5.53, 5.3, 4.95, and 4.52. The following species of the genus *Lactobacillus* were used: *plantarum* from 4 different sources, *casei*, *arabinosis* 17-5, *dorner*, and *leichmanni*. Three of the 4 *plantarum* cultures tested grew very well throughout the entire pH range while the fourth culture preferred the lower pH range. The other 4 species, *casei*, *arabinosis* 17-5, *dorner*, and *leichmanni*, grew very well throughout the whole pH range but preferred the lower pH from 5.3 to 4.52. From these data it was concluded that a pH of around 5.5 or 5.6 was suitable for the growth of all the lactobacillus tested.

The preliminary experiments had demonstrated that medium D possessed certain advantages over medium F and that both were superior to tomato juice agar which had been used in all the preliminary work for comparative purposes. It was, therefore, decided to increase the scope of the work by running additional tests on a larger assortment of media, using medium F for comparative purposes since it produced slightly fewer but larger colonies than medium D. However, if only quantitative results were desired medium D could be used.

COMPARISON OF DIFFERENT MEDIA

This work consisted of comparing the V-8 medium F and 10 different media for plating lactic acid organisms, non-lactic acid bacteria and yeasts. The V-8 medium used in this work for comparative purposes had the following composition: 500 ml. V-8 vegetable juice (filtered through 2 layers of cheese cloth), 10 g. tryptose, 5 g. lactose, 3 g. beef extract, 15 g. agar (Difco), 0.1 g. brom cresol green made up to a liter with distilled water. It was then adjusted to pH 5.7. After autoclaving the pH was 5.5 to 5.6. The pour plate technique was used and total counts and colony size were taken. The latter part of the experimental work consisted of plating samples from cucumber and kraut fermentations in 6 different media.

The following organisms were used in this experiment:

Lactic acid organisms. *Lactobacillus plantarum* (ATCC 8014), *Lactobacillus plantarum* (NRRL B-227), *Lactobacillus fermenti* (NRRL B-585), *Lactobacillus delbrueckii* (NRRL B-445), *Lactobacillus casei* (NRRL B-442), *Lactobacillus casei* (NRRL B-441), *Lactobacillus casei* (ATCC 7469), *Streptobacterium dextranicum* (NRRL B-1254), *Betabacterium vermiforme* (NRRL B-1127), *Leuconostoc mesenteroides* (ATCC 8042), and *Streptococcus faccalis*.

Yeasts. *Hansenula subpelliculosa* (Etchell's No. R Y-135), *Torulopsis caroliniana* (Etchell's No. RY-147), *Torulopsis holmii* (Etchell's No. FFL-Y-307), *Zygosaccharomyces Sp. A.* (Etchell's No. YS-590), *Torulaspora rosei* (Etchell's No. RX-8).

Non-lactic acid bacteria. *Sarcina lutea, Micrococcus pyogenes* var. *aureus, Serratia marcescens, Escherichia coli, Aerobacter cloacae, Bacillus subtilis.*

As soon as these cultures were obtained, transfers to be used as stock cultures were made as follows: lactic acid organisms—stab culture in micro assay culture agar (Difco) with the exception of *Streptococcus faecalis* which was treated like the non-lactic acid bacteria; yeasts—dextrose broth (Difco) plus 0.2% yeast extract; non-lactic acid bacteria—nutrient agar slants (Difco).

When a test was to be made, the organisms were transferred from the stock cultures to the following media: lactic acid organisms—micro inoculum broth (Difco); yeasts—dextrose broth plus 0.2% yeast extract; non-lactic acid bacteria—nutrient broth.

The organisms were incubated at the following times and temperatures:

Lactic acid organisms. All organisms except *Leuconostoc mesenteroides* were incubated at 30°C. for 48 hours. *Leuconostoc mesenteroides* was incubated at room temperature for 48 hours.

Yeasts. *Hansenula subpelliculosa, Torulopsis candida,* and *Torulaspora rosei* were incubated for 24 hours at 30°C. *Torulopsis holmii* was incubated for 3 days at 30°C. *Brettanomyces versatilis* and *Zygosaccharomyces Sp. A.* were incubated for 6 days at 30°C.

Non-lactic acid bacteria. These organisms were incubated at 30°C. for 24 hours.

Appropriate dilutions of the actively growing cultures were made and plated by the pour plate method. Duplicate plates were poured for all the lactic acid organisms.

All of the organisms were plated in the media listed in Table 1.

TABLE 1

List of media studied

Code no.	Medium	pH of medium	Reference
1	V-8	5.7	....
2	Orange serum agar	5.4	18
3	Lemon serum agar	5.2	18
4	Tryptone glucose extract[c]	7.0	Difco (*5*)
5	Nutritive caseinate[c]	6.6	Difco (*5*)
6	McCleskey's agar	6.5	16
7	Potato dextrose agar	5.6	Difco (*5*)
8	Tryptone glucose yeast extract[c]	6.8	19
9	Tomato juice agar	6.1	Difco (*5*)
10	Sabouraud's dextrose agar	5.6	Difco (*5*)
11	Thermoacidurans agar	5.0	Difco (*5*)

[c] Media contained 0.04 g. brom cresol purple indicator per liter of medium.

An additional medium was used in the yeast study. This was potato dextrose agar acidified with one ml. of a 10% solution of tartaric acid per 100 ml. of medium. This treatment lowered the pH to 3.5 + 0.1. This was assigned code No. 7.

The lactic acid organisms after plating were incubated at temperatures described as optimum in Bergey's *Manual of Determinative Bacteriology (3).* The yeasts and the non-lactic organisms were incubated at 30°C. Colonies were counted on plates showing between 30 and 300 colonies after 2, 3, and 5 days. The size of the colony was measured in mm.

In the sauerkraut fermentation, samples were taken according to a method described by Fulde *(8).* In this manner, samples could be obtained under a nitrogen atmosphere. It was also possible to obtain representative samples from the center of the fermentation.

In the cucumber fermentation, daily samples of the brine were collected from a commercial fermentation of salt-stock pickles by the method of Etchells *et al. (7).*

Platings of samples from the sauerkraut and cucumbers were made on the following media: V-8 agar, orange serum agar, tryptone glucose extract agar, nutritive caseinate agar, tomato juice agar, and thermoacidurans agar. The plates were incubated at room temperature for 2 or 3 days.

RESULTS AND DISCUSSION

The results are presented in Figures 1 to 4. Table 2 gives the diameter of subsurface colonies.

In consideration of the media studied, it appeared that the V-8 medium was the best. In most cases, after 3 days' incubation, colonies on the V-8 medium were large and easy to count and had the following characteristics: Size—0.5-1.0 mm. in diameter; color—dark green to black, having a slight fuzzy appearance around the edges of the colony; halo—characteristic distinct yellow halos appeared around the colonies in most cases. The halo was 2 to 3 times the size of the colony. Colonies on the nutritive caseinate,

TABLE 2

Diameter of colony in mm of lactic acid bacteria in various media

Media no.	*L. plantarum* (A)			*L. plantarum* (N)			*L. fermenti*		
	Days at 30°C.			*Days at 30°C.*			*Days at 40°C.*		
	2	3	5	2	3	5	2	3	5
1	0.8-1.0	1.0-1.3	1.2-2.0	*	*	0.5-1.1	0.7-1.2	0.8-1.2	0.8-1.2
2	0.8-1.0	1.0-1.2	1.2-1.8	0.2-0.6	0.6-1.2	1.0-1.5	0.5-1.2	0.8-1.5	0.8-1.5
3	0.8-1.0	1.0-1.3	1.5-2.0	0.3-0.5	0.6-1.0	1.0-1.8	0.5-1.0	0.6-1.0	0.6-1.2
4	*	0.1-0.3	0.2-0.5	0.1-0.3	0.3-0.6	0.5-1.0	+	+	0.2-0.5
5	0.6-0.8	0.7-1.0	1.0-1.5	0.2-0.5	0.3-0.6	0.7-1.1	0.3-0.7	0.3-0.7	0.5-0.8
6	0.5-0.7	0.9-1.2	1.2-1.5	0.1-0.3	0.3-0.6	0.8-1.2	0.5-1.0	0.5-1.0	0.6-1.1
7	0.5-0.6	0.7-1.0	0.9-1.2	+	+	*	0.2-0.5	0.2-0.7	0.3-1.0
8	*	0.1-0.3	0.3-0.6	*	0.1-0.2	0.3-0.6	0.2-0.8	0.3-0.8	0.5-1.0
9	0.7-0.8	0.9-1.1	1.0-1.5	*	0.3-0.8	0.8-1.2	0.5-1.0	0.5-1.0	0.6-1.0
10	0.2-0.5	0.4-0.8	0.8-1.3	*	0.1-0.3	0.2-0.5	0.3-0.8	0.4-0.9	0.5-1.0
11	0.5-0.7	0.8-1.0	1.0-1.3	0.2-0.5	0.3-0.8	0.6-1.0	0.5-1.0	0.6-1.0	0.6-1.0

* = Colonies too small to measure. + = No growth in dilutions used. (A) = *L. Plantarum* (ATCC 8014). (N) = *L. plantarum* (NRRL B-227).

tryptone glucose extract, and tryptone glucose yeast extract agars did not produce a distinct halo in many cases. It appeared as if the acid produced by the organisms could diffuse throughout the medium. This would make it difficult to differentiate acid-producing organisms from non-acid-producing organisms. The tryptone glucose extract agar and the tryptone glucose yeast extract agar produced smaller colonies than appeared on the V-8 agar medium.

Orange and lemon serum agars as well as thermoacidurans agar could support growth of the test organisms after 2 days of incubation. However, this might not have been the case if these media contained an indicator. Although some of the test organisms did not grow out as rapidly on V-8 agar (probably due to the temporary inhibitory effect of the brom cresol green indicator), the colony count at the end of 3 and 5 days was very high.

It is desirable to have a medium that is inhibitory to organisms that are usually found as active or contaminating organisms in the early part of lactic fermentations. If non-lactic organisms grow out rapidly in a medium, they would be difficult to differentiate from lactic acid-producing organisms. The data showed that the V-8 agar medium inhibited *Sarcina lutea, M. pyogenes* var. *aureus,* and *Bacillus subtilis.* None of the other media showed this degree of inhibition.

Various yeasts isolated from cucumber fermentations, when grown on the 11 media listed in Table 1, showed that no one medium was outstanding. *Torulopsis caroliniana* did not grow on nutritive caseinate agar and *Zygosaccharomyces Sp. A* did not grow on tryptone glucose extract agar, nutritive caseinate agar, McCleskey's agar, and tryptone glucose yeast extract agar.

The effect of pH of the various media must also be considered. Although no special study of this effect was made, it was observed that there was a correlation between the pH of the medium and the growth of non-lactic acid bacteria. In general, the lower the pH, the more inhibitory the medium to these organisms.

V-8 agar, orange serum agar, tryptone glucose extract agar, nutritive caseinate agar, tomato juice agar, and thermoacidurans agar were employed to enumerate organisms of the sauerkraut and cucumber fermentations in order to show how the media would function under practical conditions. The V-8 medium, tryptone glucose extract agar, and nutritive caseinate agar were chosen because they contained an indicator capable of detecting acid-producing organisms. Orange serum, thermoacidurans, and tomato juice agars were chosen because they gave relatively high counts of acid-producing organisms in pure culture. Tryptone glucose yeast extract and lemon serum agars were not used because of their similarity to tryptone glucose extract and orange serum agars respectively.

Figure 1 shows a comparison of total counts on these media in the sauerkraut fermentation. The media showed very little difference in evaluating the total count. Figure 2 indicates the numbers of acid-producing organisms. In the early part of the fermentation, both the tryptone glucose extract and nutritive caseinate agars showed acid-producing colonies while the V-8 agar did not. However, at 10 hours counts again were made of the fermentation and no acid-producers appeared on any of the media. It, therefore, appeared that the organisms that produced acid on the tryptone glucose extract and nutritive caseinate agars were not the same as

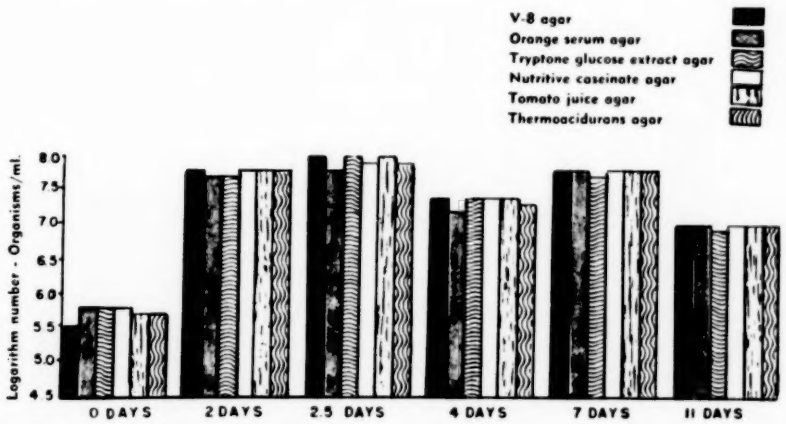

Figure 1. Evaluation of media to determine total organisms of sauerkraut fermentation.

the acid-producers appearing later in the fermentation. The organisms appearing at zero time may have been able to ferment the dextrose in tryptone glucose extract agar and some fermentable sugar in the milk of nutritive caseinate agar besides lactose. Therefore, these organisms may not have been true lactic acid bacteria.

When acid-producers first began to be noticed on the V-8 medium, gum-forming colonies appeared on the orange serum agar. These seemed to be characteristic of the leuconostoc as shown by Murdock *et al. (18)*.

On nutritive caseinate agar and tryptone glucose extract agar the acid produced by the bacteria diffused through the agar readily, often making

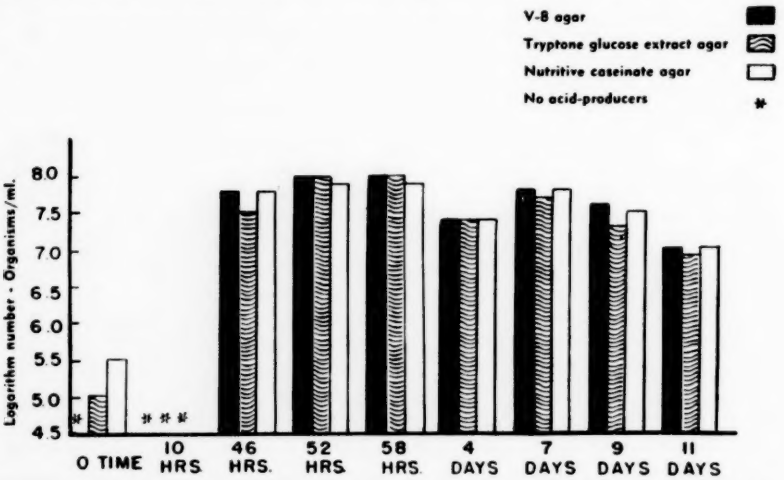

Figure 2. Evaluation of media to determine acid-producers of sauerkraut fermentation.

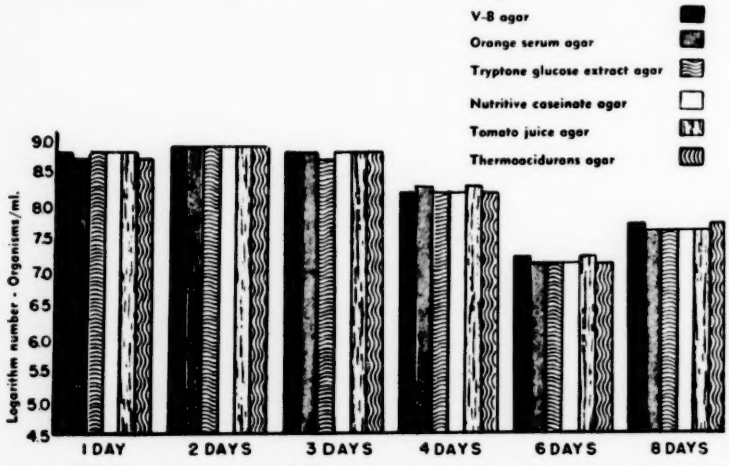

Figure 3. Evaluation of media to determine total organisms of cucumber fermentation.

differentiation difficult. In contrast to this, the acid-producing colonies on the V-8 medium were characteristically dark green with a yellow halo and easily differentiated from other organisms. The same media were used to determine total counts and acid-producers of the cucumber fermentation (Figures 3 and 4). As in the sauerkraut fermentation there was little difference between the media for enumerating total counts and acid-producing bacteria. Acid-producing colonies on the V-8 medium were characteristic as in the sauerkraut fermentation. The dark green color and yellow halo greatly facilitated differentiation from other organisms.

Since this work has been done the proposed V-8 medium has been used in making a large number of bacteriological determinations on cucumber and kraut fermentations with entirely satisfactory results. It has also been used with certain modifications in a study of the microflora of the mouth in dental caries.

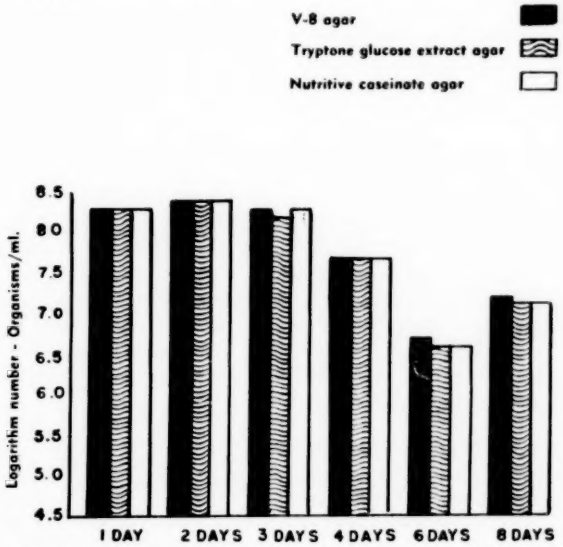

Figure 4. Evaluation of media to determine acid-producers of cucumber fermentation.

SUMMARY

1. The use of the new V-8 medium for the determination of the lactic acid type organisms (*Lactobacillus*, *Leuconostoc*, *Streptococcus*, etc.) has shown that the total counts obtained with this medium compare favorably with media commonly used for determining this group of bacteria. Preliminary tests with the V-8 medium indicated that the brom cresol green indicator had a slight inhibitory action on the lactic acid bacteria since greater total counts were obtained when it was not added to the medium. V-8 medium without the indicator also showed greater total counts than any of the other media tested. However, even with the indicator, the total counts on the V-8 medium compared favorably with those on other media tested. Although there was a greater total count on V-8 medium without the

indicator, its use in this medium is advocated in the quantity stated since the differential character of this medium is dependent upon the indicator.

There was a greater total count in the V-8 medium when dextrose was used instead of lactose. However, the difference in the total count was slight in most cases and since the colonies were much larger when lactose was used, it was decided to use lactose.

2. It has been shown that most of the lactic acid fermenting organisms give rise to a peculiar characteristic colony that appears to possess a differential character when cultivated on the V-8 medium. In comparing different media containing an indicator only the V-8 medium could differentiate between miscellaneous acid forming bacteria and the true lactic types as determined by the typical colony produced on the V-8 medium.

The appearance of the lactic organisms on the V-8 medium is somewhat variable depending on the particular organism. In general, however, the lactic organisms appear dark green to jet black. In some instances the typical yellow halo is weak or only slightly green; however, with the stronger acid producers the halo is bright yellow. Most of the lactobacilli are easily characterized by observing a bright yellow halo surrounding a jet black colony. A weaker acid producer such as *Leuconostoc* appears somewhat similar, however, the colony is generally green with weaker acid production. The above description of the lactic organisms applies only to plates with less than 120 colonies. A greater number of colonies obscures these characters and differentiation is not possible.

3. Attempts to cultivate species of the genus *Bacillus*, *Micrococcus*, and *Sarcina* have shown that the V-8 medium inhibits surface and subsurface growth. This in itself is a great advantage of the V-8 medium since it is desirable to incubate plates for the determination of the *Lactobacillus* from 2-5 days. In the past, other media have permitted "spreaders" to grow abundantly after 24-28 hours, thereby obscuring any possible determination of the lactic organisms.

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FACTORS AFFECTING THE BACTERIAL FLORA IN FERMENTING VEGETABLES[a]

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Several years ago a study was initiated to determine the nature of the bacterial changes that occur in sauerkraut fermentation. As a direct result, the causes of some of the types of poor quality sauerkraut were explained. These studies have been extended to include the bacterial fermentation of other vegetables packed either by dry salt procedures or by brining methods. The fermentations of cabbage, cucumbers, beets, turnips, beans, chard, and other vegetables have been investigated. Considerable attention has been given to the effect of various concentrations of salt and of different temperatures. Present studies on floral changes in cucumber fermentations have been motivated by the work of Etchells *et al. (1, 2, 3)* in which they have observed that better pickle stock has been obtained when cucumbers were fermented in low salt brine. This indicated an influence of type of bacterial fermentation. In recent papers, the effect of salt *(5)* and of temperature *(4)* upon the nature of the bacterial fermentation of cucumbers has been discussed. However, as will be pointed out, other factors influence the course of fermentation. Therefore the effects of the various fermenting organisms were studied by use of pure culture inoculation of cucumbers.

METHODS

The general methods of study have been described in detail in the reports mentioned *(4, 5)*. Changes in bacterial flora and acidity were determined at frequent intervals throughout the active fermentation, more often in the early stages. This ordinarily required the isolation and identification of a maximum of 400 bacterial strains from each fermentation studied.

RESULTS AND DISCUSSION

Unlike the results obtained in the studies on sauerkraut, consistent differences in quality were not obtained in studies of cucumbers or other vegetables *(4, 5)*. The numbers of bloaters, hollow pickles, or soft pickles obtained were usually too low to be significant in analysis of data. There were differences observed in cure. In general, cucumbers, beans, etc., packed in high salt brines or those fermented at low temperatures cured slowly. In several instances large numbers of the various species of bacteria isolated were attenuated in some way or other, very often in their ability to produce normal amounts of acid for the particular species. In one instance Leuconostoc strains isolated from high salt brine, failed to produce the slime typical of this species.

Development of acid in cucumber brines followed a similar pattern each season and showed the marked effect that temperature may exert upon rate of fermentation *(4)* (Figures 1, 2, and 3). In all 3 years' studies, more rapid fermentation occurred at 75°F.(23.9°C.) and above than at 65°F.(18.3°C.) and 45°F.(7.2°C.). The retarding effect of the low temperature, 45°F.(7.2°C.) upon both rate and extent of fermentation was great. An interesting observation was the apparent rapid rate of fermentation at the 3 higher temperatures that occurred at all 3 salt concentrations. The acidity in the brine rose rapidly to above 1.0% and later dropped approximately 0.2%. A theoretical

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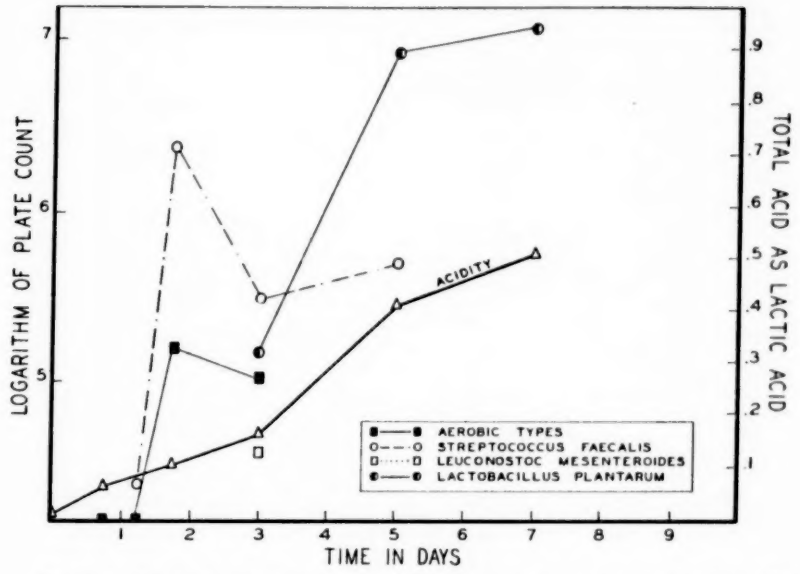

Figure 1. Change in acidity and bacterial flora in cucumber brine containing 7.5% salt, 1947 season.

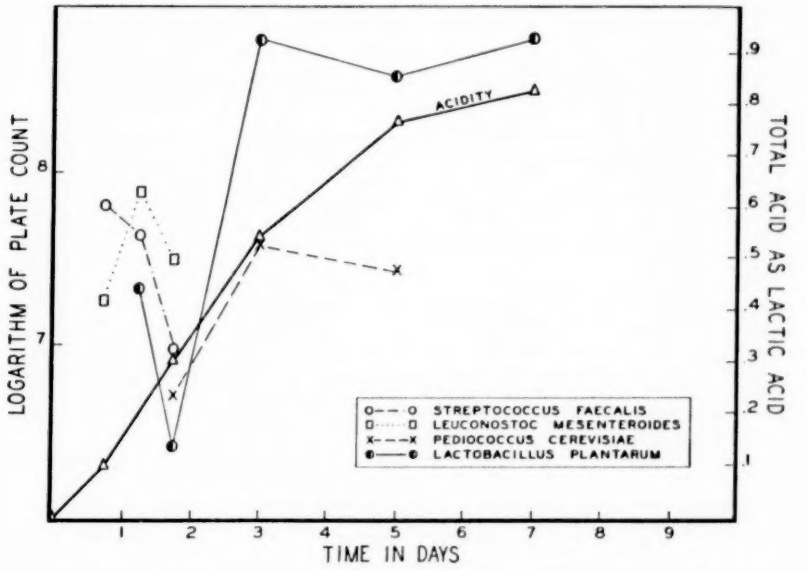

Figure 2. Changes in acidity and bacterial flora in cucumber brine containing 3% salt, 1947 season.

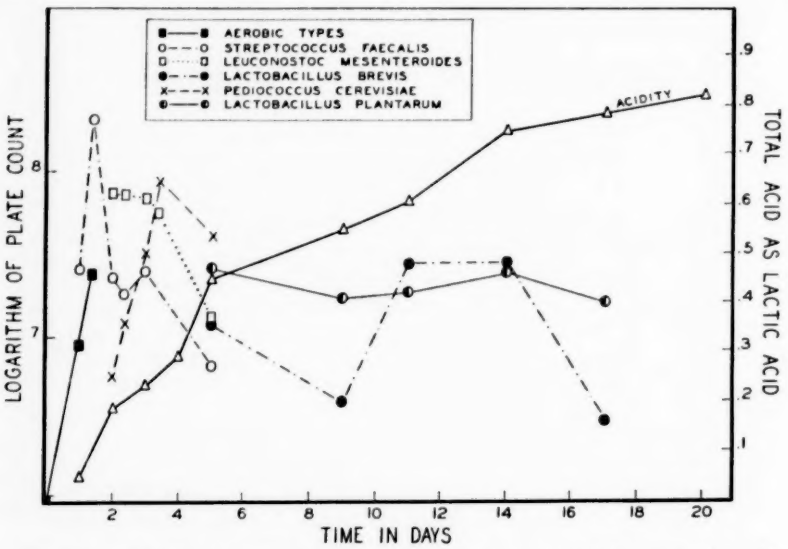

Figure 3. Changes in acidity and bacterial flora in cucumber brine containing 3% salt, 1946 season.

consideration of the situation would suggest a rapid development of acid in the brine with a slower diffusion of this acid into the cucumber. As the acid slowly diffused into the cucumber, the acidity of the brine decreased. This equilibrium of acid between brine and cucumbers may not have been attained until several days after maximum acidity was attained. Similar changes in pH were observed that may have been related to the diffusion of buffering substances. This may be an important stage in the fermentation of cucumbers.

The effect of salt *(5)* is particularly marked above a 5% level. From 2 to 5% salt, acid is produced at about the same rate. There is some retarding at 5% for the first few days. At 7½% definite retardation is effected, more so in some lots than in others. Concentrations of salt above 7½% always effected a strong inhibitory action.

The effect of salt at 3% and 7½% levels upon both acidity and bacterial flora is strikingly illustrated in representative results presented in Figures 1 and 2. In 7½% brine *Lactobacillus plantarum* and to a lesser degree *Streptococcus faecalis* appear to be responsible for the fermentation (Figure 1). This has been generally true at higher salt concentrations also. Acidity development is retarded when compared with that in 3% brines (Figure 2). In 3% brines, the species *Leuconostoc mesenteroides* and *Pediococcus cerevisiae* play an important part in fermentation. The role played by species other than *Lactobacillus plantarum* is more strikingly illustrated in Figure 3. However, again it must be pointed out that results are not constant. If one compares the results obtained under similar conditions during 3 seasons, one can note differences in predominant flora and in rate of fermentation. Acid development in the 1947 series was more rapid than in 1946. However, the part played in fermentation by various species in the 3 seasons was quite dissimilar. Similar results were obtained at the different salt concentrations used. In other words, there are factors other than salt and temperature which play an important part in the curing of cucumbers for pickles.

As previously stated, although fermentations of various vegetables by brining procedures have shown differences in rate of fermentation, the types of bacterial species and to a slight extent the end products of fermentation, little of this could be satisfactorily correlated with quality difference.

Pure culture inoculation studies were first conducted during the season of 1950. It was demonstrated that fermentation could be controlled to a marked degree by such methods, particularly when the species used for inoculation were either *Pediococcus cerevisiae* or *Lactobacillus plantarum*. Five cultures representing the species commonly isolated in previous studies were used as inocula. Forty containers were filled with No. 3 cucumbers, 10 with No. 4 and No. 5 cucumbers, and 5 with No. 2 cucumbers. These were filled with a salt brine, of such concentration, approximately 30° salometer, as to give a 3¾% salt based upon weight of water and cucumbers. These were divided into 5 lots. Each lot of 11 was inoculated with a pure culture of one of the 5 strains of lactic acid producing bacteria. A 1% inoculum of broth culture was used containing from 100 to 1,000 million cells. Each lot was again divided into 3 lots of 3 or 4 each. The first 15 lots packed were incubated for fermentation at 25°C.(77°F.), the next 20 were incubated at 18°C.(64.4°F.), and the last 20 at 32°C.(89.6°F.).

The first day plate counts made from the 15 lots that were regularly sampled were well over a million per ml. of the bacterial type desired in all samples except one. These 15 representative lots were used for plating and acidity determinations at regular intervals. Colonies were picked from the plates and identified, primarily to note how the inoculum persisted and/or with what organisms it was replaced. From the percentage isolations of each type, and plate counts, the numbers of each type were estimated during fermentation. Additional salt was not added to keep the concentration in the brine at a uniform level until after the fermentation was well under way. This required from 7 to 11 days at 89.6°F.(32°C.), 11 to 13 days at 77°F.(25°C.) and 17 to 20 days at 64.4°F.(18°C.). Additional salt was added at comparable intervals afterward until the desired concentration of approximately 60° was reached.

After the fermentation was apparently completed and curing was well under way, all lots were examined. The final pH, total acid, volatile acid, and gain or loss in weight of cucumbers was determined. In addition the percent of bloated cucumbers or those showing hollow seed areas was estimated. All cucumbers were cut to note if curing was complete.

The same methods were used in 1951 except that only No. 4 cucumbers were used and 60 jars were packed to give 4 jars for each lot. In addition, cucumbers were tested for firmness by the Ballauf pressure tester.

Results of the 1950 experiments were not conclusive. The cucumbers fermented at 89.6°F.(32°C.) and 77°F.(25°C.) were more completely cured than those fermented at 64.4°F.(18°C.). At two of the 3 temperatures, the lowest pH and the lowest acidity was obtained in the lots containing the largest cucumbers in each series. Obviously this is a buffer effect. The acidity attained varied from about 0.3 to 0.9% acid but showed no consistent relationship to inocula. The ratio between volatile and non-volatile acid was distinct and correlated with what might have been expected on the basis of the organisms used (Table 1). The two higher acid-producing homofermentative species caused average acidity ratios of .049 and .057 while the 2 heterofermentative species caused average acidity ratios of 0.174 and 0.126. These results indicated the effectiveness of inocula. However, this can be shown better by the frequency of isolation of the various species in each lot (Table 2).

From this table one may note that when *Lactobacillus plantarum* was used as the inoculum, other types did not develop. In contrast, when *Streptococcus faecalis* was used, all other types developed. These results are in line with the amount of acid each species can produce. Representative data showing the relative influence of the inoculum upon flora changes and influence upon acidity development is more clearly shown in Table 3. By such presentation of results one may obtain some idea of the total effectiveness of each species in the fermentation. A study of these data shows a floral change starting with the lower acid *Leuconostoc mesenteroides* fermentation, succeeded by a mixed flora of the next higher acid species *Pediococcus cerevisiae* and *Lactobacillus brevis* and finally by the highest acid-producing species *Lactobacillus plantarum*.

There was no extreme difference in quality of stock produced. There were a few bloaters or hollow pickles, but in only 4 lots, 3 inoculated with *Lactobacillus plantarum* and one with *Pediococcus cerevisiae*, was the number of hollow pickles quite excessive. Most of the stock gained weight during curing (Table 1). Based upon weight before refreshing, only 2 of the 11 lots inoculated with *Lactobacillus plantarum*, 2 of the 11 lots inoculated with *Pediococcus cerevisiae*, and one of the 11 inoculated with *Leucon-*

TABLE 1

Analysis at refreshing time of inoculated cucumber stock prepared in 1950

Cultures used in inoculum	Lot No.[b]	Fermented at 18°C.(64.4°F.)				Fermented at 25°C.(77°F.)				Fermented at 32°C.(89.6°F.)			
		Total acid	pH	Acetic/ lactic ratio	Gain in weight	Total acid	pH	Acetic/ lactic ratio	Gain in weight	Total acid	pH	Acetic/ lactic ratio	Gain in weight
		percent			*percent*	*percent*			*percent*	*percent*			*percent*
Streptococcus faecalis	1	.63	3.50	.142	2.8	.89	3.19	.097	7.8	.79	3.42	.097	5.5
	2	.66	3.47	.072	5.9	....	.....	.....	8.8	.89	3.38	.061	6.9
	3	.30	4.34	.254	2.5	.56	3.33	.....	2.4	.79	3.39	.085	6.5
	4	.44	3.43	.....	1.1	....	.....	.....	....	.83	3.35	.....	5.3
Leuconostoc mesenteroides	1	.43	3.69	.128	6.5	.62	3.51	.131	13.5	.47	3.58	.092	6.1
	2	.61	3.52	.109	7.4	.62	3.31	.061	8.2	.76	3.56	.109	5.8
	3	.52	3.76	.247	4.0	.60	3.22	.....	−3.4	.66	3.47	.135	6.1
	4	.53	3.55	.....	3.6	....	.....	.....	....	.75	3.36	.....	3.9
Lactobacillus brevis	1	.51	3.61	.121	6.5	.80	3.32	.154	8.8	.57	3.52	.135	6.0
	2	.61	3.52	.167	6.8	.83	3.40	.197	7.7	.58	3.61	.143	5.7
	3	.50	3.88	.348	2.0	.53	3.28	.....	1.6	.62	3.68	.129	5.8
	4	.29	3.82	.....	4.4	....	.....	.....	....	.57	3.41	.....	5.9
Pediococcus cerevisiae	1	.54	3.52	.067	0.1	.42	3.52	.091	7.2	.58	3.45	.042	5.2
	2	.58	3.51	.040	−0.9	.70	3.52	.051	8.1	.84	3.29	.051	7.8
	3	.48	3.69	.057	1.4	.63	3.30	.....	2.3	.55	3.61	.055	4.5
	4	.47	3.59	.....	−3.4	....	.....	.....	....	.76	3.20	.....	7.1
Lactobacillus plantarum	1	.46	3.51	.069	3.6	.72	3.28	.046	7.5	.86	3.28	.039	6.1
	2	.65	3.40	.055	1.6	.62	3.47	.042	2.2	.77	3.31	.045	7.0
	3	.58	3.50	.060	4.7	.49	3.26	.....	−1.3	.75	3.30	.039	7.2
	4	.45	3.49	.....	−6.9	....	.....	.....	....	.79	3.22	.....	4.5

[b] Number 3 cucumbers were used in all No. 1 and 2 lots and all lots fermented at 32°C. Number 4 and 5 cucumbers were used in No. 3 lots, fermented at 18° and 25°C., and Number 2 cucumbers were used in No. 4 lots fermented at 18°C.

TABLE 2

Effectiveness of inoculum as shown by the numbers of cultures identified

Lot No.	Species used as inoculum	Temperature of fermentation	Number of cultures identified—1950, 1951										Total number of cultures identified, excluding yeast and aerobes	
			Streptococcus faecalis		*Leuconostoc mesenteroides*		*Lactobacillus brevis*		*Pediococcus cerevisiae*		*Lactobacillus plantarum*			
			'50	'51	'50	'51	'50	'51	'50	'51	'50	'51	'50	'51
G	*Streptococcus*	18°	53	51	2	37	33	18	2	1	49	63	139	181
A	*faecalis*	25°	47	65	35	16	0	30	0	0	112	23	194	164
M		32°	28	88	10	28	4	94	0	0	130	38	168	248
H	*Leuconostoc*	18°	1	0	43	98	23	49	11	1	80	45	158	193
B	*mesenteroides*	25°	0	0	49	31	32	7	36	0	48	153	165	191
N		32°	0	0	41	34	9	17	0	1	105	139	173	191
J	*Lactobacillus*	18°	0	0	0	0	82	122	0	0	77	44	159	166
C	*brevis*	25°	1	0	0	0	64	144	0	2	129	62	196	208
O		32°	0	0	0	0	63	95	0	1	99	60	163	156
K	*Pediococcus*	18°	3	0	7	0	11	16	85	63	37	42	141	121
D	*cerevisiae*	25°	0	1	0	0	0	2	115	124	79	59	194	201
P		32°	0	2	0	0	1	2	45	91	120	96	166	193
L	*Lactobacillus*	18°	1	0	0	0	18	0	0	0	147	69	166	69
E	*plantarum*	25°	0	0	0	0	0	0	0	0	97	166	197	167
R		32°	1	0	0	0	1	2	0	0	161	175	163	177

ostoc mesenteroides were weight losses noted. Thus one can only say that differences in quality were not marked enough to be correlated with types of inoculation.

Results obtained in the 1951 season showed a marked relation between quality of stock and type of inoculum added. It was the first season that sufficient numbers of cucumbers developed hollow seed areas to make data apparently significant. Further agreement between various lots was good. Correlation between total acid, ratio of volatile to non-volatile acid, percent gain or loss in weight, percent hollow or bloated cucumbers, and the effect one might expect from the type inoculum used was very good (Table 4). Firmness readings and pH readings could also be correlated with the above observations. It should be added that the hollow or bloated cucumbers had not collapsed and that most of them were filled with brine after refreshing. Thus the actual loss or gain in weight would be more striking than recorded.

TABLE 3

Representative effects of inoculation with Leuconostoc mesenteroides and with Streptococcus faecalis upon bacterial flora and the development of acid in cucumbers in 3¼ per cent salt brines

Time in days	Total acid percent	pH	Total plate count 100,000 per ml.	Estimated number of each species of bacteria, 100,000 per ml.				
				Leuconostoc mesenteroides	*Streptococcus faecalis*	*Pediococcus cerevisiae*	*Lactobacillus brevis*	*Lactobacillus plantarum*
Series B. Inoculation, *Leuconostoc mesenteroides*, 1950, 77°F.(25°C.)								
1	.07	5.00	730	730	.....	.....	.....	.....
2	.15	4.82	660	660	.....	.....	.....	.....
3	.20	4.45	3,030	1,363	.....	1,363	304	.....
4	.38	3.98	4,190	233	.....	3,490	467	.....
5	.54	4.00	8,200	.....	.....	4,200	2,520	1,680
6	.65	3.78	4,600	.....	.....	460	.....	4,140
7	.78	3.52	3,500	.....	.....	175	1,050	2,275
8	.84	3.52	2,600	.....	.....	.....	1,430	1,170
11	.92	.....	1,020	.....	.....	.....	220	800
15	.98	3.47	200	.....	.....	.....	.....	200
Series M. Inoculation, *Streptococcus faecalis*, 1951, 89.6°F.(32°C.)								
⅙	.02	5.32	333	.....	333	.....	.....	.....
¾	.05	5.70	115	.....	115	.....	.....	.....
1.8	.14	4.42	.03	.....	.03	.....	.....	.....
2.8	.24	4.08	3,400	1,360	2,040	.....	.....	.....
4	.33	4.08	2,120	1,060	1,060	.....	.....	.....
5	.36	3.92	1,905	571	858	.....	476	.....
6	.48	3.73	3,950	.....	.....	.....	3,950	.....
7	.49	3.71	2,775	.....	.....	.....	2,775	.....
8	.56	3.69	5,000	.....	.....	.....	4,445	555
9	.64	3.70	1,900	380	380	.....	475	665
10	.72	3.61	2,750	.....	.....	.....	2,613	137
13	.85	3.47	1,445	.....	.....	.....	725	720
15	.89	3.39	1,000	.....	.....	.....	.....	1,000
27	.87	3.40	329	.....	.....	.....	.....	329

As in 1950, the inoculum in each case controlled that part of the fermentation which might have been expected (Tables 2 and 3). The data show that the highest acid-producing species when used as inoculum controlled the entire fermentation. The low acid-producing species developed and produced acid only to the extent of their acid tolerance. Then the fermentation was continued by other types.

The best stock was obtained from those lots inoculated with the heterofermentative lactic acid producer, *Leuconostoc mesenteroides*, followed closely by *Streptococcus faecalis* in which case the leuconostoc always played a part in the fermentation (Tables 2 and 3). The poorest results were obtained when *Lactobacillus plantarum*, the homofermentativ-

lactic acid producer, was used as inoculum. Inoculation with *Pediococcus cerevisiae*, also a homofermentative type, resulted in slightly better stock than that of the *Lactobacillus plantarum* inoculation. Consistently for all inoculations the total acidity and gain in weight of the pickles were lower for fermentations at 18°C.(64.4°F.) than at 32°C. (89.6°F.) and the ratio of volatile to non-volatile acidity and the percent of hollow pickles at 18°C.(64.4°F.) exceeded the values for inoculations at 32°C.(89.6°F.).

Results obtained with the 12 containers inoculated with *Lactobacillus plantarum* were quite similar. The entire fermentation was due to this species (Table 2). When compared with lots inoculated with other organisms the total acidity was lowest in all 3 temperature series, averaging 0.51%. The volatile acid ratio was lowest, the 12 averaging 0.050 (Table 4). Seven samples showed loss in weight and the hollow cucumbers ranged from 4 to 48% in the various lots, averaging 23%, the highest percent of all inoculations. These cucumbers were not as well cured as those in other series. They gave the lowest average firmness readings. The difference in firmness readings between different lots was not great.

Results obtained with the 12 containers inoculated with *Pediococcus cerevisiae* were quite similar. Early fermentation was due to the inoculated species but the fermentation was completed by *Lactobacillus plantarum* (Table 2). Both are homofermentative strains of lactic acid bacteria. Except for samples inoculated with *Lactobacillus plantarum* the total acidity was lowest in 2 of the series and the volatile acid ratio was lowest in 2 series, the 12 averaging 0.112 (Table 4), when compared with lots inoculated with other species. Eight samples showed loss in weight and hollow cucumbers ranged from 0 to 52% in the various lots averaging 21%. There was some discrepancy in results in the 4 jars fermented at 32°C.(89.6°F.). This lot showed the lowest percent hollow pickles of those inoculated with this species.

The best results were obtained with lots of cucumbers inoculated with *Leuconostoc mesenteroides* (Table 4). The inocula persisted only through the early fermentations as expected (Table 2). The flora was replaced by *Lactobacillus plantarum* and to some extent *Lactobacillus brevis*. The 8 lots at 32°C.(89.6°F.) and 25°C.(77°F.) contained no hollow cucumbers. The 4 lots fermented at 18°C.(64.4°F.) showed hollow cucumbers, the highest percentage 12. Thus the 12 lots gave an average of only 2% hollow cucumbers. All 12 lots showed some gain in weight. Each series of 4 averaged highest in total acid in the temperature series. The pH, however, was no lower in average than those lots inoculated with *Lactobacillus plantarum* in which the average acid was only 0.51%. Further, each lot of 4 gave the highest average firmness reading in the temperature series, although these results may not be too significant. The contrast between these lots and those discussed above was striking. However, it is indicative and must be repeated.

The 12 samples inoculated with *Lactobacillus brevis* were intermediate between those mentioned above in many respects. The inocula persisted longer than did the inocula of *Leuconostoc mesenteroides* but like those, the inocula was replaced by *Lactobacillus plantarum*. Results were less consistent. At 18°C.(64.4°F.) all lots showed very high volatile acid, high pH, low acidity, and high percentage of hollow cucumbers. On the other hand, lots fermented at 25°C.(77°F.) and 32°C.(89.6°F.) were lower in volatile acid and higher in acidity, and had a lower pH and lower percent of hollows. Results were more variable for lots at different temperatures than in the series inoculated with other organisms. Possibly temperatures play a greater part in the development of this organism than in that of the other 4 species.

In the 3 representative samples of the 12 inoculated with *Streptococcus faecalis*, the strains persisted over a longer period than normal (Table 3). However, *Leuconostoc mesenteroides* was isolated almost as frequently after the first day or so as in those lots inoculated with this species. Results obtained were also quite similar to those inoculated with the leuconostoc (Table 4). One could almost consider these lots as normal controls.

The fact that the results have been so striking this one season would indicate that seasonal differences in stock may vary considerably. These studies must be repeated before we can be certain of the results. However, it might be pointed out that in pickle inoculation studies conducted in barrels in 1950, poorer results were obtained in two lots inoculated with *Lactobacillus plantarum* than in uninoculated controls. Other species were not used. These results are similar to those often observed with sauerkraut

TABLE 4

Analysis of cucumbers and brine at time of refreshing. Fermented at 32°C.(89.6°F.) M-R, at 25°C.(77°F.) A-E, and at 18°C.(64.4°F.) G-L

Lot No.	Inoculum	At refreshing			After refreshing		
		Total acid	pH	Acetic/ lactic acid	Percent loss or gain in weight	Percent hollow	Firmness
	At 32°C.						
M1	*Streptococcus*	.79	3.41	.211	+2.2	0	12.9
2	*faecalis*	.79	3.47	.237	+2.6	0	14.9
3		.99	3.09	.098	+2.0	6	.....
4		.63	3.35	.053	+2.2	4	12.4
N1	*Leuconostoc*	1.05	3.22	.126	+2.2	0	15.1
2	*mesenteroides*	.93	3.21	.100	+3.0	0	15.6
3		.84	3.11	.109	+3.7	0	.....
4		1.00	3.21	.129	+2.8	0	12.8
O1	*Lactobacillus*	.62	3.36	.154	+1.4	8	14.6
2	*brevis*	.80	3.30	.107	+0.6	0	14.2
3		.78	3.08	.076	−0.5	0	.....
4		.91	3.18	.105	−0.3	4	13.0
P1	*Pediococcus*	.74	3.20	.069	+1.0	4	13.0
2	*cerevisiae*	.65	3.41	.188	−0.5	8	13.8
3		.55	3.28	.155	−0.4	0	.....
4		.69	3.38	.134	−0.5	0	13.4
R1	*Lactobacillus*	.62	3.25	.043	−1.0	15	11.8
2	*plantarum*	.67	3.22	.039	+0.2	4	13.1
3		.48	3.19	.054	+0.8	36	.....
4		.66	3.21	.040	+1.6	19	10.8
	At 25°C.						
A1	*Streptococcus*	.66	3.55	.234	+1.5	0	15.5
2	*faecalis*	.78	3.33	.109	+1.5	5	14.7
3		.68	3.31	.097	+1.0	0	.....
4		.78	3.33	.157	+1.9	4	14.8
B1	*Leuconostoc*	.96	3.21	.109	+1.8	0	16.1
2	*mesenteroides*	.67	3.50	.230	+2.0	0	15.6
3		.96	3.18	.099	+3.4	0	.....
4		.96	3.18	.112	+3.1	0	14.5
C1	*Lactobacillus*	.60	3.38	.195	+1.0	4	14.3
2	*brevis*	.72	3.32	.109	−0.9	....	16.5
3		.64	3.30	.165	−0.9	4	.....
4		.55	3.32	.163	−0.4	0	15.4
D1	*Pediococcus*	.69	3.37	.071	+1.7	6	15.2
2	*cerevisiae*	.67	3.34	.047	+2.7	27	14.7
3		.62	3.37	.110	+0.8	21	.....
4		.67	3.38	.142	−1.1	17	13.7
E1	*Lactobacillus*	.....	.....	.....	−1.0	19	12.7
2	*plantarum*	.56	3.31	.039	+0.1	12	15.6
3		.61	3.20	.036	−1.4	8	.....
4		.36	3.41	.044	−0.7	13	15.1
	At 18°C.						
G1	*Streptococcus*	.65	3.43	.149	+1.4	0	14.0
2	*faecalis*	.69	3.49	.228	+1.1	8	15.0
3		.78	3.22	.114	+1.3	10	.....
4		.67	3.28	.283	+2.0	4	14.9
H1	*Leuconostoc*	.75	3.40	.197	+1.6	6	15.5
2	*mesenteroides*	.75	3.39	.119	+1.1	6	15.0
3		.77	3.21	.096	+0.5	12	.....
4		.53	3.52	.313	+2.1	4	13.5
J1	*Lactobacillus*	.48	3.50	.303	+0.1	20	13.1
2	*brevis*	.36	3.65	.374	−1.5	25	13.8
3		.46	3.40	.295	−1.7	54	.....
4		.46	3.60	.316	−0.5	14	14.0
K1	*Pediococcus*	.36	3.42	.110	−4.1	50	13.4
2	*cerevisiae*	.42	3.41	.105	−1.8	35	15.1
3		.46	3.32	.102	−5.8	40	.....
4		.52	3.36	.102	−1.4	52	14.1
L1	*Lactobacillus*	.45	3.37	.061	+0.9	15	13.0
2	*plantarum*	.39	3.41	.074	−1.1	48	13.8
3		.39	3.26	.073	−1.5	44	.....
4		.41	3.40	.055	−1.1	41	13.7

inoculation studies in that lots inoculated with the homofermentative organisms normally resulted in poor quality sauerkraut.

These observations have not been fully explained. Curing of pickles is apparently a physical as well as chemical phenomenon connected with fermentation and enzyme activity that is definitely tied up with salt, acid, alcohol, and pectin relationships. As curing takes place, the individual cell walls become more permeable and thus equilibrium can be obtained more readily. Fermentation started by the heterofermentative lactic acid, acetic acid, alcohol, and mannitol producing bacteria seem to effect these changes in permeability more quickly than fermentation by homofermentative lactic acid bacteria.

These considerations are borne out by observations of fewer hollow pickles, more rapid cure, and higher acidity at the same pH, indicating greater diffusion of cell contents to establish equilibrium.

The 3¾% salt used in these studies is about equivalent to starting cucumbers in 30° brine. However, the salt concentration was not kept at about 30° brine level by addition of salt but was allowed to remain at a low level until the most active fermentation was practically completed. Thus, the lactic acid bacteria were not inhibited from producing the maximum acidity of which they are capable. A high acidity not only aids in suppressing the growth of undesirable microorganisms but also seems essential to good curing of cucumbers. If the heterofermentative lactic acid producing bacteria play a desirable role in this fermentation, it may be partially due to the action of carbon dioxide replacing the air and producing a more anaerobic condition. It was observed that yeast did not develop as readily on the surface of such containers as upon those inoculated with the homofermentative bacteria.

From a practical standpoint, it should be pointed out that these fermentations were started with low salt brines and additional salt was not added until fermentation had proceeded enough to have allowed marked change in acidity and in the physical structure of the cucumber. This did not occur for at least a week and possibly a month, depending on temperature and size of cucumbers. More time is necessary at low temperature and with the larger cucumbers to accomplish this change.

SUMMARY

The fermentation of vegetables whether conducted by salting or brining procedures may be brought about by a sequence of lactic acid producing bacteria. Five species have been isolated in previous studies so frequently that they are considered important in the fermentation.

Fermentation is influenced by the amount of salt and by the temperature. Other factors undoubtedly of seasonal nature play an important role in fermentation.

It has been demonstrated by pure culture inoculations with the 5 species of lactic acid bacteria commonly found in fermenting cucumbers, that the type of fermentation and quality of product is dependent upon the lactic acid bacteria involved in fermentation. It seems quite apparent that best results are secured in a fermentation started by the heterofermentative lactic acid, acetic acid, alcohol, and carbon dioxide producing species *Leuconostoc mesenteroides*. The poorest cucumber stocks were obtained from the pure homofermentative fermentations.

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PROPERTIES OF PURIFIED FUNGAL POLYGALACTURONASE

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Some of the properties of polygalacturonase preparations from commercial fungal preparations have been reported in recent investigations *(3, 4, 5, 7)*. The results of older investigations *(4)* on pH optima, stability, and activation by alkali salts are subject to errors arising from the use of ill-defined substrates and of preparations containing pectin esterase (PE) as well as pectin polygalacturonase (PG). The effect of several factors on the activity of purified PG prepared from three commercial fungal sources is presented here; the investigations in more detail have been reported by Rahman *(11)*.

EXPERIMENTAL

Source and purification of polygalacturonases. Diluent-free pectinase preparations* were used in this study. These were purified by the methods described by Lineweaver *et al. (6)*. The PG from Pectinol 45AP was obtained by adsorption on alginic acid, elution with NaCl at 5°C. followed by acid inactivation of PE, and had an activity of 3.6×10^{-2} PG units per mg. total nitrogen. The PG from Pectinol 46AP and Wallerstein pectinase, since they were both found to be only slightly adsorbed by alginic acid, were purified by acid treatment followed by ammonium sulfate and lead acetate fractionation. Their PG activities in similar units were 0.8 and 1.2, respectively.

Pectic acid preparations. Two preparations of pectic acid were used in this study, one was the Wichmann pectic acid prepared from Sunkist Growers Exchange pectin NF as described by Newbold *(9)* and Newbold and Joslyn *(10)*, the other was pectic acid prepared from the Sunkist Growers Exchange sodium ammonium pectate by the procedure described by Joslyn *et al. (2)*. The analysis of the original pectin and of the pectic acid preparations using the methods described previously *(10)* are shown in Table 1.

TABLE 1

Characterization of the pectic substances

Sample	Moisture	Ash	Methoxyl	Uronic anhydride	Neutralization equivalent	Specific optical rotation	Intrinsic viscosity
	%	%	%	%			
Pectin "NF"	7.9	1.2	10.2	84	985	.....	.....
Wichmann pectic acid	7.2	0.9	0.14	90	186	281.0	1.10
Pectic acid from sodium ammonium pectate	10.5	1.3	.....	81	184	252.0	1.125

PG activity. The PG activity was determined by the Jansen and MacDonnell *(1)* modification of the Willstätter-Schudel hypoiodite method.

Effect of pH. The effect of pH in the range of pH 3-8 in 0.1 M lactate and phosphate buffers containing 0.5% Wichmann pectic acid at room temperature was determined by measuring the rate of increase in meq of reducing groups liberated in the initial stages of hydrolysis where this was constant. The PG activity of Wallerstein

*Two of these were from the Rohm and Haas Company and designated Pectinol 45AP and Pectinol 46AP; a third diluent-free pectinase preparation used was from the Wallerstein Laboratories, Inc.

PG rose sharply from a very low activity (.005 or below) at pH 3 to an optimum in the range of pH 4-5 and then decreased more slowly and almost linearly with pH to zero activity at pH 8.0. PG from 45AP and 46AP resembled each other closely in the effect of pH on their activity, being very low or almost negligible at pH 3, reaching an optimum at pH 4.0 which was maintained to pH 5.5, and then decreasing linearly to zero at pH 7.5. The type of buffer used had no effect on PG activity. Low activity at pH 3.0 is probably due to precipitation of pectic acid at this pH, the substrate presenting relatively smaller surface to attack of enzyme. At pH 8 the enzyme is completely inactivated.

Alkali inactivation of PG. The only satisfactory method of inhibiting PG activity at various stages of hydrolysis with the least effect on the substrate and its degradation products was found to be treatment with dilute alkali. Heat inactivation was not suitable because of its effect on pectic acid and the usual protein precipitates, particularly trichloracetic acid and phosphotungstic acids, precipitated pectic acid, and its early degradation products as well as the enzyme. Matus *(8)*, using unpurified commercial pectinase, reported complete inactivation of PG in 0.5 N NaOH at 20°C. in 40 minutes. The complete inhibition of PG at pH 8.0 or higher was shown above. It was found that its inactivation by adjusting the pH of the test solution to 9.0 by addition of the calculated amount of 0.1 N NaOH and storage at 0°C. for 2 weeks or longer did not result in any noticeable change in the substrate both as regards increase in reducing power or depolymerization. The glycosidic bond, in absence of oxidation, is known to be stable to dilute alkali. The inactivation of PG by raising pH, however, is reversible.

The inhibition of PG activity at high pH value and its reversibility was repeatedly observed. Typical of the many results obtained was that with purified PG from 45AP on 0.5% pectic acid solution at pH 5.0. Aliquots of this solution were brought to 7.0-9.5, and reducing power determined immediately and after storage at 0°C. for one week and 2 weeks. At pH levels of 7-8.5, the iodine reducing power of the aliquots increased during storage, more so at 7.0 than at higher pH values, indicating incomplete inactivation. At pH 8.8-9.5, no change in reducing power was observed. Similarly when PG activity in a 0.5% pectic acid solution containing PG from 46AP was inhibited by addition of 0.1 N NaOH to pH 9.0 at various stages of hydrolysis and the reducing power determined initially and after storage at 0°C. for 2 weeks, no difference was observed.

Typical of the observed reversibility of high pH inactivation was the data obtained with PG from Wallerstein pectinase acting on 1% pectic acid solution at pH 5.0. Aliquots of this solution were withdrawn at several stages of hydrolysis, the PG inactivated by adjusting to pH 9.0, and the reducing power determined. After storage at pH 9.0 at room temperature for 3 hours, aliquots were removed, adjusted to pH 5.0, and stored at room temperature for 3 hours; a second series of the original samples was readjusted to pH 5.0 after 72 hours at pH 9.0 and then stored for 24 hours. The final reducing power in all cases was the same as that of the original solution after the same period of enzyme activity, after allowing for dilution during pH adjustment.

Effect of enzyme concentration. PG preparations from 3 sources were diluted so that their initial activities were the same and then added in varying amounts to a reaction mixture adjusted to 0.5% pectic acid in 0.05 M lactate buffer at pH 5.0. The relative activities expressed as increase in meq of reducing groups per 5 ml. aliquot per minute from the linear portions of the activity curves are shown in Table 2. With all 3 enzymes, the relative activity increased linearly with enzyme concentration in the range of 0.1 to 0.5 ml. of enzyme added and then fell off and finally approached constancy. Similar results have been reported for unpurified PG preparations *(1)*.

Effect of temperature. Ninety-nine ml. of 0.5% pectic acid substrate in 0.05 M lactate buffer at pH 5.0 were brought to the desired temperature and then 1 ml. of enzyme preparation added. Five ml. aliquots were withdrawn, after 2.5 minutes and 5 minutes, and at 5-minute intervals to 35 minutes, and added to 0.9 ml. 1 M Na_2CO_3, and immediately cooled to 25°C. with ice water. The activity was then determined as previously described. The reducing power first increased with time linearly (usually in the first 2.5-5 minutes) and then decreased less rapidly. At 75°C. in all cases for all periods of time from 2.5 minutes to 35 minutes no activity was observed. At 65°C., during the first 5 minutes the activity was low, and then increased during the period from 5 to 20 minutes and remained constant thereafter. The results obtained for crude

TABLE 2

Effect of enzyme concentration

Enzyme concentration	Relative activity		
	45AP	Pectinase	46AP
ml.			
0.1	0.005	0.0057	0.005
0.2	0.009	0.012	0.009
0.5	0.021	0.024	0.020
1.0	0.028	0.029	0.027
2.0	0.035	0.037	0.034
4.0	0.038	0.038	0.039

as well as purified enzymes are shown in Table 3. The purified enzymes were somewhat more sensitive to temperature than the corresponding crude preparations. The optimum temperature for all preparations, however, is in the range of 45-55°C.

Effect of various inhibitors. The effect of $HgCl_2$, NaN_3, NaF, ICH_2COOH, $(NH_4)_2AsO_4$, and SO_2 on activity of PG from 45AP and Wallerstein pectinase was determined in a pectic acid solution at pH 5. Monoiodoacetic acid at 1.9×10^{-2} M, NaN_3 at 3.8×10^{-2} M, $HgCl_2$ at 3.8×10^{-3} M, and NaF at 7.6×10^{-2} M had but little inhibiting effect. The slight inhibition by NaN_3 at 3.8×10^{-2} M increased somewhat at 7.6×10^{-2} M but even at this concentration was not as great as that of arsenate at 3.8×10^{-2} M. In all cases the inhibiting effect was more slight initially and more apparent after 5 to 7.5 minutes of reaction time. Sulfur dioxide inhibition increased with increase in concentration, being very slight at 1,000 p.p.m. and 2,000 p.p.m., and appreciable only at 4,000 p.p.m.

TABLE 3

Effect of temperature on PG activity

Temperature °C.	45AP (Purified)		45AP (Crude)		Pectinase		46AP	
	Relative activity[b]	% Relative activity	Relative activity[b]	% Relative activity	Relative activity[b]	% Relative activity	Relative activity[b]	% Relative activity
25	0.02	33	0.020	33	0.019	29	0.019	33
30	0.033	55	0.028	46	.....	.....	.....	.....
35	0.048	80	.....	.....	0.042	64	0.042	75
40	0.055	92	0.044	73	0.053	81	.....	.....
45	0.060	100	.....	.....	0.065	100	0.050	90
50	.....	.....	0.060	100	.....	.....	0.056	100
55	0.049	82	0.058	96	0.060	92	.....	.....
60	.....	.....	.....	.....	0.045	69	0.039	70
65	0.006	10	0.005	8	0.005	7	0.006	11
75	<0.001	.....	<0.001	.....	<0.001	.....	<0.001	.....

[b] Calculated as increase in meq. reducing groups per 5 ml. aliquot per minute and obtained from the linear portion of the activity curves.

SUMMARY

PG free from PE was prepared from 3 commercial fungal sources. All the purified PG preparations were similar in their properties, the most appreciable difference being in the range of pH at which optimum activity was observed.

The pH optimum for PG action on pectic acid was in the range of pH 4-5; the temperature optimum was in the range of 45-55°C.; and the PG activity was in general resistant or only slightly affected by the usual inhibitors.

PG activity was very slight at pH 3, and inhibited at pH 7.5-8.0. For prolonged storage, inactivation of PG activity in the presence of pectic acid and products of hydrolysis occurred at pH 9.0 and this inactivation was found to be reversible.

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CHANGES IN THE LIPID FRACTION OF VALENCIA ORANGE JUICE DURING PASTEURIZATION

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Earlier investigations at this laboratory *(3, 5)* demonstrated that changes occur in the lipid fraction of pasteurized orange juice during storage, but did not show whether any of these were due to pasteurization. Since pasteurization is a time-temperature treatment like storage, although employing a higher temperature for a shorter time, it might conceivably produce similar effects. One of the possible explanations of differences in the flavor of pasteurized as compared to the flavor of fresh orange juice might be compositional changes in the juice lipids—probably in combination with loss or change in the volatile flavoring constituents during heating; caramelization of the sugars; reactions involving sugars, acids, amino acids, ascorbic acid, and proteins.

The purpose of this paper is to compare analytical data on the composition of the lipids from fresh orange juice and from the same juice almost immediately after pasteurization.

EXPERIMENTAL

Preparation of lipids. The fresh Valencia orange juice was obtained from a local processing plant during the 1950 season. In the laboratory, it was mixed and divided into 2 equal batches. One batch was pasteurized at 90.4°C., canned, cooled quickly, and placed in storage at 2.5°C. for 2 days until the lipid could be extracted. The fresh juice was processed promptly.

Lipids were extracted from the batch of fresh juice and later from the pasteurized juice using the procedure described by Huskins, Swift, and Veldhuis *(3)*. The juice was passed through an Oliver[b] filter and the lipids were extracted from the material retained in the filter cake with acetone. This solvent was then removed by evaporation and the lipid taken up in petroleum ether. This step eliminated the water that had been extracted from the damp filter cake. The petroleum ether solution of the lipids was placed in a 3-necked flask equipped with a stirrer and condenser. After most of the petroleum ether had been distilled at atmospheric pressure, the flask was immersed in cold water, the stirrer was started, and vacuum connected. The water bath was heated slowly (45 minutes) to boiling and for 15 minutes thereafter. During this time the lipid was stirred continuously and the vacuum was maintained at 10 mm. of mercury. Ice water was circulated through the condenser and a dry ice trap was placed in the line between the vacuum pump and the condenser. During this vacuum process of drying the lipid, a small amount of fragrant volatile liquid was retained in the dry ice trap. This substance was only slightly soluble in water and had a spectral absorption maximum of 244 mμ. It was not identified.

Analytical methods. Samples of the lipids were saponified and the unsaponifiable matter was determined according to the Kerr-Sorber method. The fatty acids were extracted from the acidified aqueous residue with petroleum ether. Aliquots of the aqueous solution remaining were used for the determination of the resin acids, referred to by

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[a] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agriculture Research Administration, U. S. Department of Agriculture.

[b] The mention of equipment and products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other equipment or products having the same or similar properties.

Nolte and von Loesecke *(5)*, and for glycerol by an adaptation of an A.O.A.C. method (6th Edition, page 551, 1945). Nitrogen was determined on the whole lipid by the Kjeldahl method and the phosphorus by ashing the lipid with magnesium nitrate, precipitating the phosphorus from the acid solution of the ash as ammonium phosphomolybdate, and subsequent titration of this complex with standard alkali. Amino nitrogen was determined on an acid hydrolysate of the lipid by the Van Slyke *(10, 11)* method and choline on the same solution by the procedure of Thornton and Broome *(9)*.

The analysis of the fatty acids was made using the spectrophotometric method of Brice, Swain, Schaeffer, and Ault *(2)*. The acids studied contained a reddish-brown constituent which had been shown by Huskins, Swift, and Veldhuis *(3)*, not to interfere with this analysis.

RESULTS AND DISCUSSION

Composition of lipids. The composition of the lipids from fresh and pasteurized juice as determined by the analyses is shown in Table 1.

The most striking difference is in the lower percentage of unsaponifiable matter in the pasteurized juice lipid. It is possible that some kind of decomposition, the nature of which is unknown, may have produced water-soluble fragments of this fraction which were then lost to the aqueous phase of the juice. The sterols, high molecular weight alcohols, carotenoids, and hydrocarbons that usually are considered as making up the unsaponifiable matter do not offer a clue to the mechanism of the loss, nor does it seem likely that these constituents could contribute much to the flavor of the juice. Thus, while the possibility of a connection between flavor change and the loss of unsaponifiable matter due to pasteurization cannot be excluded, it seems probable that any heat damage to flavor occurs elsewhere in the juice, particularly since lipids seldom exceed 0.08% of the juice.

TABLE 1

Composition of the lipids from fresh and pasteurized juices by radicals and classes of compounds.

Constituent	Fresh juice	Pasteurized juice
	%	%
Unsaponifiable	19.05	16.53
Fatty acids, free	33.50	34.30
Fatty acids, total	57.55	56.53
Dienes	0.72	0.86
Trienes	0.014	0.017
Tetraenes	0.002	0.005
Arachidonic	0.000	0.000
Linolenic	4.05	3.98
Oleic	23.22	23.50
Linoleic	18.20	17.35
Saturated acids	11.21	10.83
Resin acids	10.43	10.31
Sterol glycosides	5.85	4.93
Glyceryl radical	1.63	1.40
Mono acid phosphate radical	4.06	4.34
Nitrogen total	0.68	0.73
Choline N	0.24	0.27
Amino N	0.24	0.24
Unaccounted for N	0.20	0.22
Cholinyl radical	1.78	2.02
Ethanolaminyl radical	0.74	0.74
Iodine (Wijs 30 min.)	115.26	115.02

The percentage of free fatty acids is slightly higher in the lipid from the pasteurized juice while that of glycerol is a little lower. If these small differences are significant, a slight hydrolysis of the glycerides is indicated. No hydrolysis of the phosphatides is evident from the values of phosphorus nor from those of choline, amino nitrogen, and total nitrogen.

There is little evidence of change in the composition of the fatty acids. The percent conjugated diene acids increased slightly during pasteurization while that of linoleic decreased. This possibly is evidence of slight oxidation *(1, 6)*.

SUMMARY

A comparative analysis of the lipid from fresh and pasteurized orange juices shows little change in composition. A slight loss of unsaponifiable matter was noted. It appears unlikely that changes in flavor and lipid composition, due to pasteurization, are closely related.

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THE HYDROLYSIS OF PECTIC ACID BY PURIFIED FUNGAL POLYGALACTURONASE

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Although the enzymatic hydrolysis of pectic substances has been under investigation for a considerable time, and some aspects of its mechanism are known, the nature of this hydrolysis is not yet fully elucidated *(7, 9)*. Most of the data on the hydrolysis of 1,4-glycosidic bonds of polygalacturonic acid preparations have been obtained with crude or at most partially purified polygalacturonases from commercial fungal enzymes. Jansen and MacDonnell *(4)* showed that polygalacturonase (PG) obtained from Pectinol[a] preparations and free from pectin esterase (PE) will hydrolyze pectic acid rapidly, but its rate of hydrolysis of pectinic acids of varying degree of esterification with methyl alcohol decreases with increase in esterification. They explain the differences in rate of hydrolysis and in extent of hydrolysis by postulating that both the carbonyl groups adjacent to a 1,4-linkage must be free for it to be labile to PG. Matus *(11)*, however, reported extensive glycosidic splitting of highly esterified pectic acids by crude PG preparations obtained from Swiss commercial filtration enzyme preparations. The hydrolysis of pectic acid by PG preparations from Pectinols has been followed usually by determination of decrease in viscosity and increase in free aldehyde groups; these preparations are characterized by extensive decrease in viscosity (of the order of magnitude of 50%) when the extent of hydrolysis as measured by increase in reducing power is 2% or slightly less *(4, 7, 9, 10)*. Hydrolysis apparently occurs by random scission of the glycosidic linkages of pectic acid leading to the accumulation of D-galacturonic acid. By the application of chromatographic methods, compounds intermediate between pectic acid and D-galacturonic acid have been detected during the course of hydrolysis of pectic acid by PG preparations from three different molds *(6)*, and also of pectinic acid by commercial pectic enzyme preparations *(16)*. The formation of lower molecular weight products of hydrolysis has been demonstrated, also by the use of ion exchange resins *(3)*. The polygalacturonase of *Neurospora Grassa* has been recently shown to convert more than 50% of the pectic acid hydrolyzed to a low molecular weight polyuronide (M W ca. 4,000), *(18)*; di- and tri-galacturonic acids have been isolated among the end products of hydrolysis of pectic acid by the polygalacturonase of *Saccharomyces fragilis (13)* and also by a commercial fungal preparation *(1)*. Fungal preparations thus may differ in their content of PE, and contain more than one PG enzyme (pectin depolymerase as well as pectin polygalacturonase).

Since but little information was available in 1948 at the start of our investigations *(14)* on the course of hydrolysis of well defined pectic acid

[a]Commercial pectic enzyme preparations manufactured by Rohm and Haas Company, Philadelphia, Pennsylvania.

by purified fungal PG, the hydrolysis of 2 pectic acid preparations by 3 purified PG preparations at different initial concentrations of substrate and of enzyme was followed by determining decrease in alcohol precipitable pectic acid, in calcium-precipitable pectic acid, in viscosity and specific optical rotation and increase in reducing power, and in D-galacturonic acid content. The results of these investigations are reported here.

EXPERIMENTAL MATERIALS AND METHODS

PG was prepared from Pectinol 45AP, Pectinol 46AP, and Wallerstein Pectinase by the methods described by Lineweaver *et al. (8)*. These preparations were quite similar in general properties *(15)*, and for the first two were shown to be monospecific so far as kind of bond is concerned and heterospecific so far as molecular size of polygalacturonic acid is concerned *(8)*. Two pectic acid preparations were used as substrate, one the so-called Wiehmann pectic acid obtained from Sunkist Growers Exchange orange pectin NF and the other a pectic acid obtained from the Exchange sodium ammonium pectate. These preparations are described elsewhere *(12, 15)*.

Alcohol-precipitable matter was determined on aliquots of the enzyme-substrate mixture whose PG was previously inhibited by adjustment of pH to 9.0 *(15)*, by adjusting the pH to 5.0 or 7.0, precipitating with 80% ethanol, filtering on a sintered glass funnel, washing, and drying at 70°C. in a vacuum oven. It includes the enzyme protein as well as higher molecular weight polygalacturonic acids. The calcium pectate content was determined as described by Newbold and Joslyn *(12)*. The viscosity of aliquots brought to 0.15 M NaCl was measured with 5-ml. Ostwald pipettes of 80- to 90-second efflux time at 27.3°C. The optical rotation was measured in a Schmidt and Haensch polarimeter using a 1-dm tube and sodium lamp. The reducing power was determined by the method of Jansen and MacDonnell *(4)*. The molecular weights were calculated from measurements of osmotic pressure and specific intrinsic viscosity by methods described elsewhere *(12)*.

Mono-galacturonic acid content was determined by a modification of the naphtho-resorcinol procedure *(2)*. Roboz *(17)* did not find her modification to be reliable for measurement of galacturonic acid content of pectin hydrolyzates. Our modification, however, was not only reproducible but also was unaffected by the non-alcohol and calcium-precipitable lower polymers of galacturonic acid in the enzyme hydrolyzed pectic acids. At the time we used it, pure preparations of di- and tri-galacturonic acids were not available to us; but subsequently Phaff and Luh *(13)* confirmed our observations. The procedure used was as follows: an aliquot of the hydrolysate was diluted so that the final one-ml. sample used in the determination contained 20-100 mg. of galacturonic acid. Only in this range is the optical density of the pink color, given by naphtho-resorcinol and galacturonic acid under the specified conditions, proportional to the concentration of galacturonic acid. To one ml. of this solution, in a 25-ml. graduated cylinder, 2 ml. of freshly prepared 0.5% naphtho-resorcinol (1,3-dihydroxynaphthalene)[b] in 50% alcohol solution and 2 ml. of concentrated HCl were added slowly. After mixing, the cylinder was transferred to a water bath maintained at 50°C. ±1°C. for exactly 90 minutes. The sample was then left to cool for 10 minutes. Then 20 ml. of absolute ether was added and the mixture was thoroughly shaken for 1 minute. The bottom layer was siphoned off, and a pinch of anhydrous Na_2SO_4 was added to remove traces of water in the ether fraction. The optical density of the pink color developed on shaking was read immediately at a wave length of 600 mμ (No. 60 filter) in a Klett-Summerson photoelectric colorimeter, using ether as a blank. The galacturonic acid content was then computed from a standard curve obtained with a pure sample of galacturonic acid. For exact determinations, however, it is advisable to run controls consisting of pure galacturonic acid every time. It was found that concentration of naphtho-resorcinol reagent, temperature, and duration of heating considerably affect the intensity of the color formation. Also, the color developed initially on shaking tends to fade on standing, giving low results. Hence, it is absolutely essential to standardize this method with respect to concentration of naphtho-resorcinol reagent, temperature, and time of heating.

[b] Obtained from A. D. Mackay, New York.

Hydrolysis of Wichmann pectic acid. The course of hydrolysis of Wichmann pectic acid in 0.05 M lactate buffer at pH 5.0 at several levels of enzyme and pectic acid concentration is shown in Tables 1, 2, 3, and 4. Table 1 represents the course of hydrolysis of pectic acid at a concentration of 0.466% on ash-free dry weight basis by Wallerstein PG at a concentration of 0.015 mg. of total enzyme nitrogen per ml. Table 2 represents the course of hydrolysis of pectic acid at a concentration of 0.89% on ash-free dry weight basis by Wallerstein PG at a concentration of 0.0075 mg. of total enzyme nitrogen per ml. Table 3 represents the course of hydrolysis of pectic acid at a concentration of 0.89% on ash-free dry weight basis by PG from Pectinol 45AP at a concentration of 0.002 mg. of total enzyme nitrogen per ml. Table 4 represents the course of hydrolysis of pectic acid at a concentration of 0.455% on ash-free dry weight basis by PG from Pectinol 45AP at a concentration of 0.002 mg. of total enzyme nitrogen per ml.

Under the experimental conditions used, the ratio of enzyme to pectic acid concentration, 0.84 and 3.3 mg. of enzyme nitrogen per g. of pectic acid was too high to define the initial conditions of hydrolysis in the first two cases. Even after 2 minutes of reaction the extent of hydrolysis, as measured by increase in reducing power, varied from 6 to about 36%. The data presented in Table 1, for an enzyme-N : pectic acid ratio of 3.3, indicate that mono-galacturonic acid occurs early in the course of hydrolysis, but in concentrations lower than those calculated from the increase in free aldehyde groups. Its production in the first 10 minutes is lower than calculated but later is faster, although it levels off at a value considerably below the values calculated from increase in reducing power. In Table 2 there is an indication that mono-galacturonic

TABLE 1

Hydrolysis of pectic acid by PG in pectinase preparation

Sample	Time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	Free galacturonic acid mg./ml.	Calcium pectate mg.%	80% Alcohol ppt. at pH 5.0 mg.%
1	0	.....	.....	0.00	510	495
2	1	0.85	19.0	0.20	280	450
3	2½	1.64	36.7	0.67	nil	400
4	5	2.65	59.4	1.35	nil	315
5	10	3.35	75.1	2.20	nil	240
6	20	3.84	86.1	2.95	nil	130
7	30	4.12	92.3	3.40	nil	slight ppt.
8	40	4.30	96.4	3.75	nil	no ppt.
9	60	4.48	100.4	4.05	nil	no ppt.

TABLE 2

Hydrolysis of pectic acid by PG in Wallerstein pectinase preparation

Sample	Reaction time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	Free galacturonic acid mg./ml.	Specific optical rotation	Calcium pectate mg. %	80% Alcohol ppt. mg. %
1	0	.....	.....	.....	281.0	970	930
2	2	0.56	6.3	0.00	267.5	860	930
3	8	2.42	27.2	0.72	214.6	110	850
4	24	4.66	52.3	1.65	162.0	nil	760
5	64	6.03	67.0	3.40	109.0	nil	570
6	96	7.43	81.4	5.10	87.6	nil	390
7	144	8.50	95.5	7.15	67.3	nil	150
8	288	8.80	98.8	7.70	64.0	nil	slight ppt.
9	480	8.94	100.4	8.05	58.2	nil	slight ppt.

Substrate: Wichmann Pectic Acid. Concentration 0.89% at pH 5.0.
Enzyme: Wallerstein Pectinase preparation.

acid does not form in the early stages of hydrolysis, at least at 6% or less. The increase in reducing power at an enzyme-N : pectic acid ratio of 0.84, the accumulation of galacturonic acid after a slight initial lag parallel to that of increase in aldehyde groups, and the rapid initial decrease in specific optical rotation paralleling the increase in aldehyde groups are shown there (Table 2).

The changes occurring during the initial conditions of hydrolysis are better shown in Tables 3 and 4. In Table 3, for an enzyme-N : pectic acid ratio of 0.22 (the PG from 45AP is similar to that from Wallerstein pectinase [*15*]) the lag in appearance of galacturonic acid is quite evident, none occurring up to 4% hydrolysis and only traces occurring at 8.6% hydrolysis. At 80% decrease in viscosity and below no galacturonic acid was formed, indicating that appreciable depolymerization and scission of glycosidic bonds had to occur before its appearance. Jermyn and Tomkins (*6*) observed that galacturonic acid was first definitely visible on the paper chromatogram when the scission was about 25% complete and thereafter increased in amount until it was finally the only degradation product visible. Doubling the enzyme-N : pectic acid ratio, 0.44, decreased the lag in production of galacturonic acid as shown in Table 4. Here galacturonic acid first occurred at 7% hydrolysis, after the viscosity had decreased by about 88% of its initial value.

Hydrolysis of pectic acid prepared from sodium ammonium pectate. This pectic acid was not subjected to acid treatment during preparation and consequently was not as degraded and also had a higher non-uronide matter content than did the Wichmann pectic acid. Its hydrolysis by purified PG from Pectinol 46AP at pH 5.0 at a concentration of 0.875% on a dry weight ash-free basis and an enzyme concentration of 0.012 mg. of enzyme-N per ml., representing an enzyme-N : pectic acid ratio of 1.37 mg. of enzyme-N per g. of pectic acid is shown in Table 5. The hydrolysis of a 1.60% pectic acid solution by PG Pectinol 46AP at a level of 0.016 mg. enzyme-N per ml., an enzyme-N : pectic acid ratio of 1.0 is shown in Table 6. The hydrolysis of a .44% pectic acid solution by PG from 46AP at a level of 0.008 mg. of enzyme-N per ml., an enzyme-N : pectic acid ratio of 1.82 is shown in Table 7. Tables 8 and 9, respectively, show the course of hydrolysis of 1.70% pectic acid by PG from Pectinol 45AP at levels of 0.015 and 0.017 mg. of enzyme-N per ml., representing an enzyme-N : pectic acid ratio of 0.88 and 1.0, respectively. It is seen that the course of hydrolysis of this pectic acid is similar to that of the Wichmann pectic acid. In Table 5 it is seen that galacturonic acid first occurs at 8.6% hydrolysis when the viscosity has decreased to 92% of its initial value. In Table 6, under approximately similar conditions, an attempt was made to fractionally precipitate the products of hydrolysis by alcohol. The degradation products of higher molecular weight precipitable at lower concentrations of alcohol decrease more rapidly than those precipitable at higher concentration. After over 21% hydrolysis the 50% alcohol precipitate is decreased by half; this is true for the 60% alcohol precipitate at 45% hydrolysis, and for the 70% and 80% alcohol precipitate at over 60%

TABLE 3

Hydrolysis of pectic acid by purified PG in 45 AP

Sample	Reaction time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	Monogalacturonic acid mg./ml.	Calcium pectate mg.%	80% Alcohol ppt. mg.%		Specific optical rotation	Relative viscosity	% Viscosity change
						pH 2.0	pH 7.0			
1	0	.....	.....	.....	980	915	935	280.6	1.589	0
2	½	0.124	1.4	0.00	955	910	940	271.0	1.370	38.2
3	1	0.249	2.8	0.00	940	910	930	262.3	1.240	60.9
4	2	0.368	4.1	0.00	865	860	920	253.5	1.148	76.9
5	5	0.775	8.6	0.02	610	.....	900	238.7	1.058	92.6
6	10	1.470	16.3	0.10	380	350	865	214.7	1.031	97.3
7	30	2.978	33.1	0.65	nil	270	790	174.3	1.020	99.3
8	1 hr.	3.500	38.8	0.94	nil	.....	.....	158.8	1.016	100.0
9	12 hrs.	3.750	41.6	1.13	nil	205	765	145.4	.....	.....

Substrate: Wichmann Pectic Acid. Final concentration 0.9% ash-free dry weight.
Enzyme: Purified PG of 45AP.

TABLE 4

Hydrolysis of pectic acid by purified PG in 45 AP

Sample	Reaction time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	Mono-galacturonic acid mg./ml.	Calcium pectate mg. %	Relative viscosity[c]	% Viscosity change
1	0	.....	.....	.....	495	1.263	0
2	1	0.04	0.9	0.00	480	1.190	29.3
3	2	0.14	3.0	0.00	465	1.130	53.4
4	5	0.32	7.0	0.02	380	1.042	88.7
5	10	0.76	16.7	0.17	210	1.018	98.4
6	16	1.08	23.7	0.36	95	1.014	100.0
7	30	1.68	36.9	0.62	nil	.....	.....
8	1 hr.	1.93	42.2	0.82	nil	.....	.....
9	2 hrs.	2.20	48.3	1.02	nil	.....	.....
10	4 hrs.	2.37	52.1	1.16	nil	.....	.....
11	10 hrs.	2.88	63.3	1.62	nil	.....	.....

[c] Relative viscosity given for 1:1 diluted samples and at NaCl concentration of 0.15 M.

TABLE 5

Hydrolysis of pectic acid by PG in 45 AP

Sample	Reaction time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	Free galacturonic acid mg./ml.	Calcium pectate mg. %	80% Alcohol ppt. (pH 7.5 ±0.2) mg. %	Relative viscosity at 25°C. (original conc. 0.875%)	% Viscosity change	Specific optical rotation
1	0	.....	.....	.....	920	925	3.926	0	252.3
2	1	0.46	5.2	0.00	795	925	1.690	78.0	236.7
3	2	0.75	8.6	0.03	650	915	1.285	92.3	216.4
4	3	1.10	12.6	0.24	400	895	1.175	96.1	205.7
5	5	1.81	20.7	0.47	180	865	1.126	97.8	188.4
6	10	2.79	31.8	0.86	nil	830	1.098	98.8	154.0
7	15	3.24	37.0	1.15	nil	790	1.093	99.1	142.1
8	30	3.74	42.7	1.60	nil	710	1.081	99.7	133.3
9	60	4.05	46.2	1.94	nil	650	1.074	100.0	126.4

TABLE 6

Hydrolysis of pectic acid by PG from 46 AP

Sample	Reaction time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	Mono-galacturonic acid mg./ml.	Specific optical rotation	Calcium pectate mg. %	Alcohol precipitate mg. %. Concentration of alc.			
							50%	60%	70%	80%
1	0	.....	.....	.....	278.6	1750	1670	.....	1675	1685
2	½	1.01	6.3	0.08	245.0	1120	1460	1565	1655	1675
3	1½	3.37	21.0	0.80	201.0	390	985	1410	1520	1625
4	3	5.30	33.1	1.64	176.2	nil	.....	.....	.....	.....
5	5	6.30	39.1	2.05	158.0	nil	180	900	1265	1460
6	10	6.73	42.0	2.60	151.0	nil	.....	.....	.....	.....
7	30	7.24	45.2	3.00	138.8	nil	125	865	1160	1390
8	60	8.06	50.3	3.70	133.7	nil	.....	.....	.....	.....
9	2 hr.	9.33	58.3	4.75	116.2	nil	130	580	950	1315
10	10 hrs.	14.55	90.9	12.60	59.8	nil	.....	.....	.....	.....
11	20 hrs.	15.45	96.5	14.55	57.5	nil	Slightly turbid		115	480

TABLE 7

Hydrolysis of pectic acid by PG in 46 AP

Sample	Reaction time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	80% Alcohol ppt. at pH 7.0 mg. %	Relative viscosity at 25° C. (concentration 0.44%)	% Viscosity change	Specific optical rotation
1	0	.....	.....	490	2.364	0.0	252.0
2	1	0.66	15.0	473	1.097	96.4	187.2
3	2½	1.31	29.8	455	1.068	98.7	144.0
4	5	1.63	37.0	424	1.063	99.0	119.1
5	10	1.80	40.9	416	1.061	99.1	117.3
6	20	2.00	45.4	388	1.056	99.5	107.3
7	40	2.35	53.4	346	1.056	99.5	101.6
8	60	2.60	59.1	290	1.050	100.0	88.7

hydrolysis. Increasing the enzyme N : pectic acid ratio by about 50%, noticeably increased the rate of initial changes, as shown in Table 7. Tables 8 and 9 indicate that 45AP preparation was similar in its action to 46AP. In Table 8 no lag in galacturonic acid production is evident, although the quantity found was small at below 13.5% hydrolysis. Again a marked drop in viscosity occurred before the appearance of galacturonic acid was noticeable. Fractionation of alcohol precipitable matter by controlling the pH at which precipitation occurred indicated a more rapid decrease in alcohol precipitate at pH 2 during the course of hydrolysis than at 7 or 9. The galacturonic acid content of the alcohol filtrates and precipitates was determined and it was noted that appreciable occlusion of galacturonic acid occurred at all pH values, but was more marked at the higher pH values. Table 9 represents the changes during the first 30 minutes in more detail but in general confirms the data of Table 8.

Molecular weight changes during hydrolysis. The weight-average molecular weight, M_w, calculated from measurements of intrinsic viscosity of aliquots of pectic acid hydrolysate at several early stages of hydrolysis of Wichmann pectic acid by purified PG from Pectinol 45AP, under the conditions given for Tables 3 and 4, and the number-average molecular weight, M_N, calculated from measurements of osmotic pressure, are tabulated in Table 10. Both M_w and M_N decrease with increase in per cent of hydrolysis, but the decrease is less than that in relative viscosity. Since the pectic acid used as a substrate was not homogeneous, the observed changes in molecular weight represent average conditions only. The data, however, do not indicate any regular pattern of

TABLE 8

Hydrolysis of pectic acid by purified PG in 45 AP

Sample	Reaction time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	Monogalacturonic acid mg./ml.	Calcium pectate mg. %	Relative[d] viscosity at 27.3°C.	% Viscosity change	Specific optical rotation
1	0	.....	.....	0.00	1850	2.14	0	251.5
2	½	0.95	5.6	0.06	1416	1.17	85.7	236.0
3	1	2.30	13.5	0.08	484	1.08	94.6	204.8
4	2	3.99	23.4	0.87	132	1.06	96.4	175.6
5	5	5.78	34.0	1.60	32	1.04	98.2	150.8
6	10	6.29	37.0	1.98	nil	1.04	98.2	144.2
7	30	6.85	40.3	2.30	nil	1.04	98.2	136.8
8	60	7.70	45.3	2.88	nil	1.04	98.2	129.4
9	2 hrs.	9.12	53.6	4.12	nil	1.03	99.1	112.5
10	10 hrs.	14.60	85.5	12.20	nil	1.02	100.0	62.4
11	20 hrs.	15.56	91.5	14.00	nil	1.02	100.0	59.5

[d] Relative viscosity given for 1.1 diluted samples and at NaCl concentration of 0.15M.

Hydrolysis of pectic acid by purified PG in 45 AP (Part II)

Sample	80% alcohol ppt. mg. %			Alcohol filtrate fraction mg. galacturonic acid/ml.			Alcohol ppt. mg. galacturonic acid/ml.		
	pH			pH			pH		
	2.0	7.0	9.0	2.0	7.0	9.0	2.0	7.0	9.0
1	1695	1755	1765	0.06	nil	nil	.....	.....	.....
2	1515	1725	1740	0.48	0.20	0.18	0.46	0.76	0.70
3	1140	1670	1695	1.49	0.38	0.44	0.78	1.88	1.90
4	830	1630	1660	2.54	0.83	0.74	1.48	3.12	3.12
5	510	1565	1585	4.84	1.64	1.39	0.83	4.13	.....
6	440	1525	1550	5.68	2.07	2.06	0.67	4.28	4.33
7	305	1495	1540	6.26	2.37	2.06	.....	4.55	4.65
8	255	1430	1470	7.02	3.20	2.68	.....	4.59	4.80
9	230	1295	1340	8.46	4.08	3.61	.....	.....	4.33
10	no data	no data	no data	no data	no data	no data	no data	no data	no data
11	120	515	565	.....	11.30	.....	.....	2.79	.....

TABLE 9

Hydrolysis of pectic acid by PG in 45 AP

Sample	Reaction time (min.)	△ Mg. galacturonic acid/ml.	% Hydrolysis	Free galacturonic acid mg./ml.	Specific optical rotation	Calcium pectate mg. %	80% Alcohol ppt. mg. %
1	0	.....	.....	0.00	.....	1910	1960
2	2½	3.09	17.9	0.51	217.1	820	1920
3	5	5.52	32.1	1.62	170.5	32	1855
4	10	7.18	41.7	2.58	145.3	28	1715
5	15	7.45	43.3	2.80	139.5	24	1688
6	30	7.76	45.2	3.10	136.2	24	1655
7	50	8.07	46.9	3.34	131.8	20	1635

Substrate: $NaNH_4$ Pectate. Concentration 1.72% ash-free dry weight.
Enzyme: 45AP.

scission. The weight-average molecular weight gives equal significance to equal weights of very small and very large particles, and does not indicate the relative scission of small and large particles. The molecular weights shown were calculated from the equilibrium values and are higher than those calculated by extrapolation to zero time by about 800-1,100 units. Dialysis of lower molecular weight constituents occurred during osmotic pressure measurements, and this was particularly noticeable in samples hydrolyzed to 25% or over. Similar observations were made by Jansen *et al. (5)* and Newbold and Joslyn *(12)* for pectic acids of low particle size.

DISCUSSION

The data presented on the course of hydrolysis of pectic acid by purified fungal PG preparations indicates random scission of polygalacturonide chains resulting in the formation of intermediates of lower molecular weight and eventually leading to D-galacturonic acid as the end product of hydrolysis. The presence of non-uronides in the preparations of pectic acid used probably as constituents of the chain itself *(12)* should provide linkages of different hydrolytic cleavage. That these linkages are attacked first is shown by the marked depolymerization as measured by decrease in viscosity with the production of only a small amount of reducing aldehyde groups, as found by Jansen and MacDonnell *(4)* and others, in the initial stages of hydrolysis. Changes in molecular weight initially, however, are smaller

TABLE 10

Molecular weight of pectic acid and its early degradation products

% Hydrolysis	% Decrease in viscosity	Mw	Mn
0	0	24,000	.....
1.4	38.2	18,200	.....
2.8	80.9	13,600	.....
4.1	76.9	9,700	.....
8.6	92.6	4,900	.....
16.3	97.3	3,100	.....
33.1	100.0	2,200	.....
38.8	.....	1,900	.....
0	0	25,000	10,300
0.9	29.3	20,900	8,500
3.0	53.4	15,800	7,200
7.0	88.7	6,900	6,100
16.7	98.4	3,700	4,600

than the change in viscosity would indicate. The fact that D-galacturonic acid does not occur in these initial stages when the enzyme-substrate level is low enough indicates that scission of the larger molecules occurs before hydrolysis of these to galacturonic acid. The production of pectic acids of molecular weight too low to be precipitable by calcium occurs early in the course of hydrolysis; at high enzyme-N:pectic acid ratios this occurs when the hydrolysis measured by increase in free aldehyde groups is less than 36%; usually more than 90% of the calcium pectate that could be obtained is lost before hydrolysis has proceeded to 25%. At very low concentrations of enzyme, the calcium pectate content decreases progressively in the range of 0-20% hydrolysis and almost disappears at 25% hydrolysis. The mean weight average molecular weight of the products of hydrolysis which no longer are precipitable by calcium is in the range of 2,000-3,000. Similar observations were made by Newbold and Joslyn *(12)*, and Phaff and Luh *(13)* reported that neither pure di- nor tri-galacturonide was precipitable by calcium.

Between 20 and 60% hydrolysis the increase in reducing power is considerably greater than the liberation of D-galacturonic acid. Thereafter and particularly in the range of 60-90% hydrolysis the production of galacturonic acid almost parallels that of increase in reducing power. At 90 to 100% hydrolysis the increase in reducing power is almost entirely due to the hydrolysis of simple galacturonides, possibly the di-galacturonide.

At low levels of PG activity, the hydrolysis appears to be limited to depolymerization of pectic acid without liberation of D-galacturonic acid. At such low levels, hydrolysis, under the conditions described, never proceeds beyond 40 to 50%. D-galacturonic acid appears to be formed by hydrolysis of highly degraded polygalacturonic acid chains. At this stage, however, there is an occasional overlapping of 2 processes, removal of single uronic acid units and hydrolysis of polyuronides. At high levels of PG activity it is impossible to detect these stages since 10 to 20% hydrolysis

occurs in too short a time interval and galacturonic acid appears much earlier.

The quantity of alcohol-precipitate (at 80% alcohol concentration) decreases progressively until at about 90% hydrolysis the precipitate formed is negligible for a 0.5% pectic acid solution at pH 5.0. It is apparent, that all the liberated galacturonic acid together with a small amount of lower polymers of galacturonic acid (possibly di-galacturonide) are in the alcohol-soluble fraction. However, the amount of alcohol-precipitate, at any particular concentration of alcohol, from a reaction mixture, is a function of both pH and the original concentration of pectic acid. At pH values between 7 and 9, where galacturonic acid or its polymers are present as sodium salt, the amount of alcohol precipitate is practically the same. But at pH 2.0, the free acids are more soluble in alcohol of a given concentration, and the amount of precipitate is much less than at pH 7 or 9. Although no definite conclusion can be reached from experiments on alcohol precipitation at different pH values and at different alcohol concentrations, certain general remarks can be made. The decrease in alcohol precipitate at pH 7.0 and 80% alcohol concentration seems to be due mostly to the galacturonic acid liberated in the reaction mixture during the successive stages of hydrolysis. Reducing values determined on the alcohol-soluble fractions for this pH and alcohol concentration support this view. On the other hand, during the initial stages of hydrolysis, where depolymerization is the main phenomenon, and where little or no liberation of galacturonic acid occurs, there is practically no decrease in alcohol precipitate. Thereafter, it steadily decreases with increase in galacturonic acid content.

The degradation of pectic acid by PG could be correlated with the degree of bond scission as measured by specific optical rotations and iodine reducing values. Both methods are suitable for following the activity of PG preparations. The specific optical rotations approach that of galacturonic acid ($[a]_D^{20} + 53.4°$) when hydrolysis is complete.

Viscosity fell rapidly before any appreciable rise in reducing power occurred. It is apparent that more than 95% of the viscosity change occurred before the extent of hydrolysis had proceeded to 10%. Apparently depolymerization is the main phenomenon during the early stages of hydrolysis.

As already indicated, appreciable amounts of galacturonic acid are liberated after 90-95% viscosity change has occurred, i.e., after the polygalacturonic acid chain has been highly degraded.

With active PG preparations, the viscosity method is not suitable for following the hydrolytic activity. The viscosity method is, however, suitable for detecting very low concentrations of the enzyme, such as occur in cultures of several microorganisms.

SUMMARY

The course of hydrolysis of 2 pectic acids by purified PG prepared from 3 fungal sources was studied.

The course of hydrolysis was followed by the determination of changes in total pectic substances (calcium and alcohol precipitable matter) and by increase in reducing power and mono-galacturonic content. Changes in viscosity, specific optical rotation and osmotic pressure were also measured.

During enzyme action, viscosity dropped more rapidly than did the increase in reducing value. The viscosity usually dropped over 50% when less than 2% hydrolysis occurred. Mono-galacturonic acid first appeared in significant amounts when the calcium precipitable pectic acids were practically absent and increased gradually towards the later stages of hydrolysis. At later stages of hydrolysis, or in the presence of high concentration of enzyme, increase in galacturonic acid almost paralleled that of total reducing value indicating that the hydrolysis occurred first as depolymerization of large chains, and then as random cleavage of smaller molecules. Molecular weight determination by viscosity and osmotic pressure measurements supported this conclusion.

Experiments at low concentrations of enzyme showed the effects on the properties of pectic acid of the cleavage of a small percentage of its glycosidic bonds, the complete course of events was then deduced by comparison with the results from experiments using high concentration in which the earlier events took place in less than 3 minutes. The results of experiments with intermediate concentration of enzyme showed many of the features observed with both low and high concentration, but failed to give the results at the extreme ends of the range. With very low concentration of the enzyme the hydrolysis never proceeded beyond 50 to 60% even after several days.

For PG activity measurement, methods which follow the increase in reducing power and decrease in specific optical rotation are satisfactory. For indicating very low concentrations of the enzyme as they occur in cultures of certain microorganisms, the decrease in viscosity and in calcium pectate content are more suitable. Activity measurements by following the decrease in alcohol precipitate and increase in mono-galacturonic acid offer no particular advantage over others and are only suitable for indicating intermediate concentration of PG.

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